INSIGHTS INTO THE ROLE OF KISSPEPTIN AND SEX STEROID HORMONES IN THE CONTROL OF REPRODUCTION AND METABOLISM

by

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ABSTRACT

The sex steroidal hormones Estrogen (E2) and Testosterone (T) have been conclusively shown to control reproduction and development of secondary sex characteristics in both male and female vertebrates. From infancy to adulthood, E2 and T play crucial roles in the proper function of the reproductive success of all animals, most notably of course being that of humans. Years of research and studies using knock out gene animal models have identified the key modulators of E2 and T function. The gonadotrophs of the anterior pituitary synthesize and secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which regulate gonadal development, gametogenesis and the synthesis and secretion of the gonadal steroid hormones. LH and FSH secretion is primarily regulated by gonadotropin-releasing hormone receptor (GnRHR) and its GnRH ligand secreted from neurons of the rostral hypothalamus. Recently, kisspeptin (KISS1)/kisspeptin receptor (KISS1R) signaling in GnRH neurons has been shown by our group and others to play an essential role in HPG axis function. However, whether kisspeptin signaling via the Kiss1r could also regulate reproductive function at the level of pituitary is not yet established. We hypothesized and show in this thesis that KISS1R in the pituitary is integral to the proper function of the pituitary. KISS1 signaling in conjunction with the workings of the HPG axis are able to exert powerful effects on the reproductive capabilities of vertebrates. New findings however have begun to implicate sex steroid signaling in functions such as the control of metabolic parameters like glucose homeostasis, adiposity, and energy expenditure. In the clinic, metabolic diseases and infertility as often observed together. Defective E2 and T signaling is now being implicated in clinical cases of metabolic syndrome, polycystic ovarian syndrome (PCOS), and type 2 diabetes (TD). Changes in metabolic function are observed in response to gonadectomy, or normal changes such as puberty, menstrual cyclicity and menopause. However, the signals that link reproductive status and metabolic function are not well understood. Because the liver is a major metabolic organ, we hypothesized that hepatic E2/ESR1 signaling regulates proper hepatic function, including the regulation of KISS1 hormonal signals that may serve as a link between the reproductive

axis and metabolic function. Finally, we successfully modeled PCOS like metabolic defects of hyperandrogenemia in female mice, by implanting pellets of dihydrotestosterone (DHT) in these mice. We then correct the glucose and energy metabolic defects by ablating the androgen receptor in the central nervous system of these mice. These findings offer new insights as well as potential targets into the ability of sex hormones, mediated by Kisspeptin and androgens, to control not only reproduction but also metabolism.

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TABLE OF CONTENTS

Curriculum vitae

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Sex Steroid Hormones: Estrogen (E2) and Testosterone (T) Synthesis and Regulation by the HPG Axis and Kisspeptin

Sex Steroid Hormones: Estrogen (E2) and Testosterone (T)

The blood borne hormones estrogen (E2) and testosterone (T), classically known as the "sex steroid hormones," have been conclusively studied and shown to have powerful effects on the sexual development and function of vertebrate physiology (Couse & Korach, 1999). The influences of steroid hormones can be ascertained from the earliest stages of fetal life throughout adulthood. Both the control of adult reproductive organ function as well as the development of secondary sex characteristics are the result of E2 and T action. In females, the estrous cycle, development of the ovaries, and maternal behaviors are all under the control of E2 action (Koos, 2011; Porter, 1974; Simpson et al., 2005). In males, the proper development of the gonads, the proper maturation of sperm, as well as mating behaviors have all been attributed to the action of T.(Gottlieb, Lombroso, Beitel, & Trifiro, 2005; Quigley et al., 1995) The tissue targets of E2 and T are numerous and varied since their receptors are widely distributed, thus the actions of both E2 and T must be considered in both males and females.

Beyond their canonical roles in reproduction, a growing body of evidence suggests that E2 and T also influence metabolism and contribute to glucose homeostatic functions. Clinical evidence in women has shown that hypoestrogenemia in postmenopausal women leads to increased obesity and cardiovascular risk,(Gruber, Tschugguel, Schneeberger, & Huber, 2002; Soderqvist, von Schoultz, Tani, & Skoog, 1993) and hyperandrogenemia results in Polycystic Ovarian Syndrome (PCOS)(Goodarzi, Dumesic, Chazenbalk, & Azziz, 2011; McCartney & Marshall, 2016). Males can also suffer from defective hormone signaling. Men with estrogen insensitivity have been shown to be at a greater risk of osteoporosis, to have decreased glucose tolerance, and to have increased levels of circulating insulin (Faustini-Fustini, Rochira, & Carani, 1999; Rochira & Carani, 2009). Although clinical studies suggest that sex steroid hormones play crucial roles in metabolic functions, the mechanisms of how sex steroid hormones regulate reproductive and metabolic function are not yet fully understood. One aim of this thesis is to bridge that gap in knowledge by studying the role of E2 in males as well as

the role of T in female metabolism under conditions of androgen excess (referred to as hyperandrogenemia).

Regulation of Estrogen and Testosterone by Hypothalamic Pituitary Gonadal Axis (HPG)

The control of reproduction is the responsibility of the hypothalamic-pituitary-gonadal axis (HPG) as diagrammed in Figure 1.1. At the top of this axis are neurons of the hypothalamus which secrete gonadotropin-releasing hormone (GnRH) in a pulsatile manner (Clarke & Cummins, 1982). The synthesized hormone travels along the neuronal axon and is released into the median eminence, which reaches the GnRHR on the pituitary gland to stimulate the synthesis and release of two factors—LH and FSH. Both LH and FSH then enter the blood circulatory system before binding to their receptors—on the ovaries in females and on the testes in males (Baird, Swanston, & Scaramuzzi, 1976; Leipheimer, Bona-Gallo, & Gallo, 1984). E2 and T are primarily synthesized at the gonadal level. As will be expanded on later, there is hormonal feedback in females—and to a lesser extent in males—which controls the timing and amount of E2 and T production. GnRH is released in a pulsatile manner from the hypothalamus into the hypophyseal portal system of microcirculation at the base of the brain, after which it binds to GnRHR on the surface of gonadotrophs of the pituitary gland (Nippoldt, Reame, Kelch, & Marshall, 1989). The binding of GnRH to GnRHR elicits intracellular calcium signaling that leads to the secretion of LH and FSH from the pituitary gland (Baird et al., 1976). Both of these gonadotropins then reach the gonads by general circulation.

In males, LH causes the release of T from the Leydig cells of the testes (Rochira & Carani, 2009). The HPG axis assists to fine tune the total amount of T in circulation, which is accomplished by T negatively feeding back on pituitary LH production and hypothalamic GnRH secretion in order to reduce the levels of circulating T. Furthermore, FSH has been shown to stimulate secretion of the hormone inhibin from sertoli cells (Makanji et al., 2014). In turn, the presence of inhibin reduces FSH secretion from the anterior pituitary.

In females, hormonal regulation involves the interplay of both negative and positive feedback in order to facilitate the essential rise and fall of E2 levels in reproductive female physiology. E2 levels vary through the menstrual cycle, with levels highest near the end of the follicular phase just before ovulation (Clarke & Cummins, 1982). Feedback occurs at the ovarian theca cells and granulosa cells where progestins and inhibin are respectively produced (Watson & Stacy, 2010). Similar to its actions in males, increases in inhibin levels result in decreased FSH output from the gonadotroph cells of the pituitary gland. Increased levels of E2 and progesterone synthesis and secretion have been observed in the early to mid-follicular phase of the female menstrual cycle. This increase serves to inhibit LH and FSH secretion from the pituitary.

As the menstrual cycle progresses from the follicular to the luteal phase, there is a rise in LH and FSH levels leading to a decrease in E2 production. The most notable change in LH and FSH levels is seen during the preovulatory surge. There is a neuroendocrine switch which toggles from a negative to a positive feedback loop, causing E2 levels to rise dramatically and allowing for follicular ovulation to occur. The effects of increased positive feedback are further amplified by increased sensitivity to GnRH stimulation by the LH secreting pituitary gonadotrophs (Homburg, 2005). However, this form of signaling is not always infallible. Defective feedback signaling has been noted in the clinical setting as well as in mice models. For example it is unsurprising to find that in women with PCOS, increased GnRH release is a marker of metabolic syndrome. Such an increase in GnRH release causes alterations in LH and FSH levels, leading to irregular menstrual cycles (Blank, McCartney, & Marshall, 2006; Shayya & Chang, 2010). The fine tuning of the female menstrual cycle is demonstrative of the powerful ability hormonal feedback has to control hormone synthesis.

Kisspeptin

Of particular interest in this thesis is the role of the secreted peptide kisspeptin (KISS1) in the regulation of the HPG axis. While originally discovered in 2003 by the cancer field, when it was called metastin (Lee et al., 1996), KISS1 has been primarily studied by reproductive

neuroendocrinologists due to its clear and powerful effects on GnRH neurons (Cravo et al., 2011; Iwata, Kunimura, Matsumoto, & Ozawa, 2017). Kisspeptin-expressing neurons reside in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of the hypothalamus, and send neuronal projections to the GnRH neurons (Pasquier et al., 2014). It should be noted that only female hypothalami contain an ARC. KISS1, through its G-protein coupled receptor KISS1R (formerly GPR54), has been shown to be a key regulator of pubertal onset via the homeostatic regulation of the HPG axis and generation of the preovulatory LH surge.

It has recently been shown by our group that disruption of Kiss1R in GnRH neurons in mice results in failure to progress through puberty due to hypogonadotropic hypogonadism (Novaira, Ng, Wolfe, & Radovick, 2009). This demonstrates that KISS1 is essential to the initiation of gonadotropin (LH/FSH) secretion at puberty. KISS1/KISS1R are expressed in a wide range of reproductively active tissues in the brain, pituitary, ovary, and testes, however they are also expressed in metabolically active tissues within the liver, pancreas, and fatty tissues (Ohtaki et al., 2001). In the mouse, hypothalamic (central) Kiss1 expression is acutely regulated by E2 signals, which are mediated by the E2 receptor (ESR1) (Wintermantel et al., 2006). The role and regulation of KISS1 within non- hypothalamic (peripheral) sources is only beginning to be understood. In Chapter 2 we provide evidence for a role of the pituitary KISS1R in mediating signals to the pituitary and thus affecting its function. Utilizing cre-lox recombinant technology we ablated KISS1R in the pituitary gonadotrophs, creating a new mouse model termed PKIRKO (Pituitary Kisspeptin Receptor Knockout).

Work from our group has shown that the liver is a source of circulating KISS1 and that hepatic KISS1 serves to regulate glucose stimulated insulin secretion (Song et al., 2014). Considering the important role of hypothalamic KISS1 in regulating reproductive function, these results raise the possibility of interplay between the metabolic and reproductive functions of KISS1. However, little is known about the function and regulation of hepatic KISS1. In

Chapter 3 we propose that E2 directly regulates hepatic KISS1 expression as well as the expression of other gluconeogenic genes.

Steroidogenesis: Estrogen and Testosterone Biosynthesis

The majority of E2 and T is derived from the carbon-19 steroid hormone cholesterol (Mendoza-Hernandez, Calcagno, Sanchez-Nuncio, & Diaz-Zagoya, 1984). The synthesis of either E2 or T depends not only on the tissue type (for example, testicular versus ovarian tissue), but also on the cellular location (for example, mitochondria vs. smooth endoplasmic reticulum) of a series of enzymes which ultimately convert cholesterol into active metabolites such as mineralocorticoids, glucocorticoids, estrogens (E2), androgens (T), or progestogens (Figure 1.2). Although each group of the aforementioned metabolites of cholesterol conversion are important to vertebrate physiology, of most relevance to this thesis is the biogenesis of estrogens and androgens.

In females, E2 and T synthesis begins in the mitochondria of theca interna cells of the ovary, where a protein called stAR facilitates the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Chang, T. Y., Chang, Ohgami, & Yamauchi, 2006; Miller, 2007). Once inside the inner mitochondrial membrane, the synthesis of androstenedione from cholesterol by the enzymes 17α-hydroxylase and by 17β-hydroxysteroid dehydrogenase (17β-HSD) is able to occur (Pollow, Lubbert, & Pollow, 1975). The gene Cyp17 is the source of 17α-hydroxylase. The main role of androstenedione is to serve as a precursor for the synthesis of both androgens and estrogens. In the female ovary, androstenedione travels from the theca cells into granulosa cells. It is inside granulosa cells where androstenedione is converted via aromatase to three estrogenic compounds (Mindnich et al., 2005). Those compounds are 17β-estradiol, estrone, and estriol. In terms of estrogenic potency, 17β-estradiol is the most potent estrogenic compound, and is followed by estrone and estriol (Miller & Auchus, 2011).

It is important to note that during pregnancy, a fourth type of estrogen called estetrol is produced by the fetal liver (Holinka, Diczfalusy, & Coelingh Bennink, 2008). Estetrol is

generally considered a weak hormone because its levels are only detectable during pregnancy, and because there are low affinities for its receptors, those being Estrogen receptor alpha (ESR1) and E2 receptor beta (ER-β) (Warmerdam, Visser, Coelingh Bennink, & Groen, 2008). In addition the corpus lutea, a structure that is maintained in the ovary during pregnancy, produces an important reproductive hormone called progesterone (Baulieu & Schumacher, 2000). Progesterone is catalyzed from pregnenolone via 3β-hydroxysteroid dehydrogenase action in the corpus luteum (Pang, 2001). Smaller amounts of progesterone are also produced by the adrenal glands during mineralocorticoid synthesis. Progesterone has been referred to as a "pregnancy hormone" because of its strong actions on female anatomy during pregnancy. Those actions include inhibiting lactation in the mammary glands until postpartum as well as inhibiting the movement of uterine smooth muscles during delivery (Patel et al., 2015).

The production of female T results from the conversion of androstenedione to testosterone by 17β-HSD from theca cells (Miller & Auchus, 2011). The compartmentalization of aromatase and 17β-HSD in vertebrates allow for the endogenous dual production of both E2 and T. The aromatase gene *CYP19* encodes for the aromatase enzyme and is found in numerous tissues in the body including those of the brain, breast, adipose, muscle and bone (Bulun et al., 2003). This peripheral expression of aromatase also allows for E2 production in males, as will be expanded on below. The importance of aromatase to steroidogenesis has been well highlighted in knockout mouse models and in studies of aromatase inhibition. Such studies conclusively show that both aromatase knockout in mice (Fisher, C. R., Graves, Parlow, & Simpson, 1998; Jones et al., 2001) and pharmacological inhibition (Ferretti et al., 2006) of aromatase lead to underdeveloped external genitalia/uteri (Ryan, 1982) and hypoestrogenism (Rochira, Balestrieri, Madeo, Spaggiari, & Carani, 2002), or estrogen deficiency (Smith et al., 1994). Interestingly, metabolic disturbances such as obesity (Fisher, C. R. et al., 1998), elevated lipid levels (Simpson et al., 2005), and impaired glucose metabolism have been noted in these aromatase knockout mouse models. The development of metabolic syndromes and disorders

associated with infertility are a key area of interest and study for both our lab and many other research institutes.

While E2 is typically considered to be a primarily female hormone, the respective conversion of testosterone and androstenedione to estradiol and estrone is also possible in males due to the expression of aromatase by peripheral tissues (Miller & Auchus, 2011). In the male reproductive tract, aromatase is highly expressed in the Leydig cells, Sertoli cells, and spermatocytes. The osteoblast and osteoclast cells of the bone as well as the adipocytes of adipose tissue are also sites of E2 production in males (Simpson et al., 2005) . Male E2 and/or ESR1 knockout animal models have yielded interesting but conflicting results. Thus, E2 action in males must be considered and further studied.

As noted in figure 1.2, there are other androgen metabolite precursors which also circulate in the bloodstream, such as dehydroepiandrosterone (DHEA) and dihydrotestosterone (DHT). DHEA is produced not only in the ovary but also in the zona reticularis of the adrenal glands. DHT is primarily produced in peripheral tissues (skin and prostate) via the conversion of T to DHT by the enzyme 5α-reductase. We and other labs utilize DHT (Andrisse et al., 2017) in animal studies of androgen action because of its inability to be converted to E2 by aromatization and higher binding potency to androgen receptors (AR) (Burger, 2002), thus allowing for the sole analysis of androgen signaling. As is already well understood, both T and DHT play major roles in the development of male sexual characteristics (Luetjens & Weinbauer, 2012). Such sexual characteristics include the development of male reproductive tissues (such as those of the testes and prostate), increases in muscle and bone mass, and the growth of body hair (Mooradian, Morley, & Korenman, 1987). Within the blood circulatory system, over 98 percent of E2 and T is bound to either albumin or sex hormone binding globulin (SHBG). Free unbound sex steroids make up about 2 percent of all sex hormones found in vertebrates (Luetjens & Weinbauer, 2012). The free unbound hormones are able to directly enter cells to affect their action.

Nuclear Receptor Signaling: Estrogen Receptors (ESR1/Erα, ESR2/Erβ and GPER)

For E2 to cause changes in target cells, it must first activate estrogen receptors (ER) inside of the cell. This is accomplished by binding to ERs known as estrogen receptor alpha (ESR1/ERα) and estrogen receptor beta (ESR2/ERβ) (Green et al., 1986). Of recent is the discovery of a non-genomic ER signaling pathway mediated by membrane localized G protein-coupled estrogen receptor 1 (GPER1, GPR30) (Liu et al., 2009). ESR1 and ESR2 behave like most classic steroid receptors by directly navigating the cell lipid membrane in order to enter the cytosol (Kumar, Green, Staub, & Chambon, 1986). After dimerization, ER binds E and enters the nucleus, directly binding to estrogen response elements (EREs) and regulating the transcription of the target gene. EREs have been shown to be evolutionary conserved among chordates and contain the consensus sequence of 5'GGTCAnnnTGCACC-3' (Driscoll et al., 1998). Both ESR1 and ESR2 have a zinc finger type motif in the DNA binding domain (DBD) in conjunction with an N-terminal transactivation domain (AF1) allowing for further stabilization of ERs to their target promoters (Mangelsdorf et al., 1995; Nilsson et al., 2001). A c-terminal ligand binding domain (LBD) allows for binding with co-factors and other transcription activating proteins (Nilsson et al., 2001). Ultimately, it is this protein-DNA complex in conjunction with chromatin and histone modifications that affect transcription of ER regulated genes.

Known differences between ESR1 and ESR2 include the sharing of only 56% percent of the DNA-binding domain (DBD) (Monroe et al., 2005), as well as that ESR1 elicits the proliferation of cell lines, while ESR2 inhibits such proliferation (Faulds, Olsen, Helguero, Gustafsson, & Haldosen, 2004; Strom et al., 2004). Besides these structural differences, there are also vast differences between in tissue distribution between ESR1 and ESR2. Expression of ESR1 can be found in the ovarian theca cells, uterus, breast, bone, Leydig cells, white adipose tissue, pituitary gland, adrenal gland, heart, and brain. Expression of ESR2 is predominantly found in the ovarian granulosa cells, vascular endothelium, lungs, and testes. In addition, the

liver expresses ESR1 and GPER1, while ESR2 expression is undetectable (Prossnitz & Barton, 2014).

In general, the study of E2/ESR1 signaling has been limited to its roles as a reproductive influencer, and their roles in metabolism are understudied. Knockout models of ERs have been conducted to implicate their roles in both males and females. Lubahn et al, produced mice lacking ESR1 and termed them αERKO (Lubahn et al., 1993). They found that both sexes of αERKO are infertile due to the lack of ovulation in females and disrupted mating behavior in males. Furthermore, they found that both sexes had decreased bone weight and density. These were just a few of the physiological consequences of the deletion of ESR1. Deletions of ESR2 yielded milder results. Krege et al have shown in their ESR2 knockout mice that while females were sub fertile, the males remained fertile and displayed normal mating behavior (Krege et al., 1998) The mammary glands in female αERKO mice were described as being immature, while those of ESR2 knockouts had normal structure and normal lactation.

Several studies in both sexes have shown that the liver is an E2 responsive organ (Ciocca & Roig, 1995; Fisher, B., Gunduz, Saffer, & Zheng, 1984; Francavilla et al., 1986). In males, emerging studies—including our labs finding that hepatic ESR1 is crucial for regulation of hepatic gluconeogenesis and lipid metabolism in males—indicate that ESR1 signaling is important for homeostasis (Qiu et al., 2017). Knockout studies of E2 in males showed that perturbation of ESR1 resulted in obesity (Della Torre et al., 2016; Stubbins, Holcomb, Hong, & Nunez, 2012), metabolic dysfunction (Gallagher et al., 2007), and increased risks of chronic diseases (Keene et al., 2008). A seminal study was conducted by Hewitt and colleagues in which they demonstrated that in mice in which the aromatase gene has been deleted (ArKO), the development of hepatic steatosis could be normalized by estrogen treatment in male knockout mice but not female knockout mice (Hewitt, Pratis, Jones, & Simpson, 2004). Furthermore male mice with liver-specific ESR1 knockout display increased accumulation of fat in the liver as well as irregular insulin signaling under high-fat diet (HFD) feeding (Zhu, Martinez, Emfinger, Palmisano, & Stafford, 2014).

In post-menopausal women, there is a gradually increasing deficiency in estrogen levels. Recent data has suggested that these women are more likely to suffer from mitochondrial dysfunction, cellular senescence, declining immune responses to injury, an in-balance between antioxidant formation, and oxidative stress (Brady 2015). All these defects during menopause dramatically affect the liver. Liver physiology and morphology is altered in post-menopausal women. The volume, blood flow, and function of the liver decrease by approximately 1% per year after about age 40 to 50 (Iber, Murphy, & Connor, 1994). Furthermore, liver volume decreases by 20% to 40% in an elderly person, with this reduction being more significant and noticeable in women (Uebi, Umeda, & Imai, 2015). Unfortunately, progression of nonalcoholic fatty liver disease and hepatocellular carcinoma have been shown to be more severe in post-menopausal women (Yang et al., 2014).

Taken together, these aforementioned studies suggest that estrogen plays a protective role in both sexes. Uebi et al concluded that E2 regulates liver protein turnover, supports hepatocyte proliferation, and is highly involved in hepatic recovery after injury (Uebi et al., 2015). Knowing the clear impact of E2 on hepatic success, studies such as ours discussed in Chapter 3 provide further insights into the action of E2/ESR1 signaling in the liver. In Chapter 3 we provide evidence for the E2 regulation of the hepatic hormone Kisspeptin.

Nuclear Receptor Signaling: Androgen Receptor

The actions of T on responsive cells and tissues are mediated by NR3C4 (nuclear receptor subfamily 3, group C, member 4), also known as the androgen receptor (AR). AR expression can be found all over the body in both males and females. In humans, AR mRNA and protein can be detected in the brain, reproductive tissues, bone marrow, lung, muscles, liver, pancreas, and the gastrointestinal tract. The mRNA of AR was cloned by Liao et al in 1988 (Chang, C. S., Kokontis, & Liao, 1988) and the crystal structure was resolved in 2000 by a team of researchers in Germany and Portugal (Matias et al., 2000). AR contains the conserved N-terminal domain, DNA-binding domain, and androgen-binding domain in order to regulate target gene transcription (Moras & Gronemeyer, 1998). These recent advancements in the

knowledge of AR structure has further solidified its actions as a classic steroid receptor hormone. Behaving in a similar manner as ER, AR can bypass the hydrophobic lipid membrane bilayer in order to dimerize in either the nucleus (Genomic AR signaling) or the cytosol (Nongenomic AR signaling).

Classical genomic signaling of AR acts as a steroid-hormone activated transcription factor. Once inside the nucleus, AR binds to androgen response elements (ARE) (De Bruyn, Bollen, & Claessens, 2011). The ARE is composed of two 5′-AGAACA-3′-like motifs, which are separated by three bps. (Claessens et al., 1996) Due to their proximity to promoters of androgen responsive genes, ARs are able to recruit and effect transcription machinery to ultimately regulate the target gene. As aforementioned, the ligand for AR is T, however its metabolite DHT has a greater affinity for AR than T. The EC50 of DHT for activation of the AR is 0.13 nm, while conversely the EC50 of T for AR activation is 0.66 nM. (Wilderer, 2010) Thus, it is not surprising that bioassays of AR activation reveal that DHT is overall more potent than T (Grino, Griffin, & Wilson, 1990).

Although not as clearly defined as the genomic actions of AR, there is growing evidence that AR can act through non genomic pathways. As reviewed by Lu et al, both membrane and cytoplasmic AR signaling can exert powerful effects on cellular activity (Freeman, Cinar, & Lu, 2005).At the level of the cell membrane AR has been postulated to bind to an unidentified G-protein couple receptor leading to rises in intracellular calcium (Benten et al., 1999). When not bound by heat shock protein 90 (hsp90) (Heemers & Tindall, 2007), AR in the cytoplasm is able to bind to intracellular molecules such as Proto-oncogene tyrosine-protein kinase Src—otherwise known as c-Src (Wheeler, Iida, & Dunn, 2009). Auricchio et al demonstrated that the c-Src SH2 (Src Homology 2) domain binds to a proline rich stretch of AR leading to activation of the tyrosine kinase complex (Migliaccio et al., 2000). The ability of AR to behave in non-classical steroid hormone signaling pathways suggests that T/DHT signaling must be further analyzed to understand its role in various tissues.

In Chapter 4 we propose that AR in the central nervous system is a mediator of DHT effects on glucose and energy metabolism in female mice. We utilized cre-lox technology to delete AR expressed specifically in the central nervous system. We termed our knockout mice SYNARKO (Synapsin AR knockout). As shown in Figure 1.3, we have developed a DHT regiment in order to model hyperandrogenemia in female mice. It is important to note that this low dose DHT model allows us to study AR signaling without having to compensate for the effects of obesity. We show that DHT effects on glucose intolerance and energy expenditure are ameliorated by ablating AR in the central nervous system, thus further implicating the importance of sex steroids in instances of metabolic syndrome.

Summary

Both E2 and T are crucial to the proper development and maintenance of the physiology of male and female reproduction. The HPG axis along with the secreted hormone Kisspeptin have been shown to control and regulate E2 and T's roles in reproduction. There is, however, mounting evidence that E2 and T's roles are not limited to the control of reproduction. In the clinic, comorbidity between metabolic diseases and infertility is often observed. Infertility caused by Type 2 Diabetes or by PCOS is commonly associated with metabolic dysregularities such as insulin resistance. However, the signals that link reproductive status and metabolic function are not well understood. Work from our lab and others have implicated that both E2 and T signaling contribute to the metabolic homeostasis of animals. We first defined a novel role for peripheral KISS1/KISS1R signaling action in the pituitary and the liver. We demonstrated that KISS1 is indeed involved in both reproductive and metabolic functions. Finally, we used knockout mice models that use homologous recombination in embryonic stem cells in order to analyze the effects of the absence of E2 and T on the on the reproductive and metabolic physiology of the animals. We further modeled the pathophysiological conditions of hyperandrogenemia in order to elucidate the role of sex steroids in metabolic defects. It is my hope that these studies could lead to the identification of novel therapies for a range of

reproductive disorders associated with metabolic issues such as PCOS, type 2 diabetes and other metabolic related syndromes.

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FIGURES

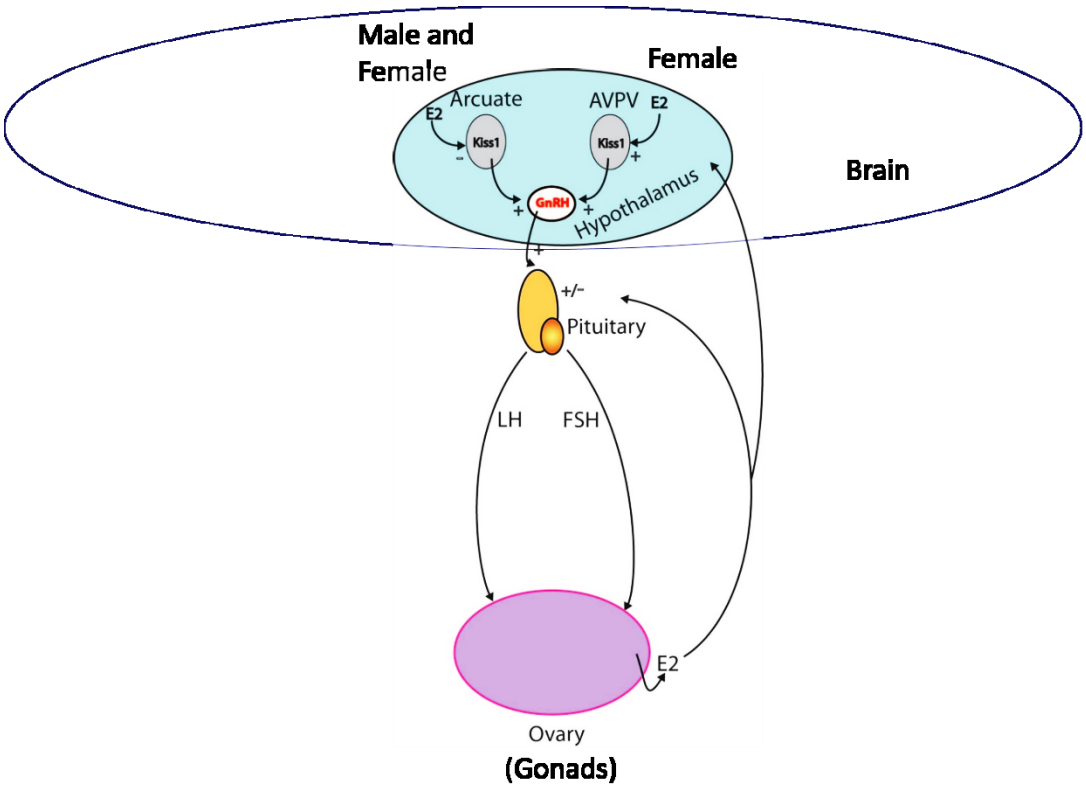
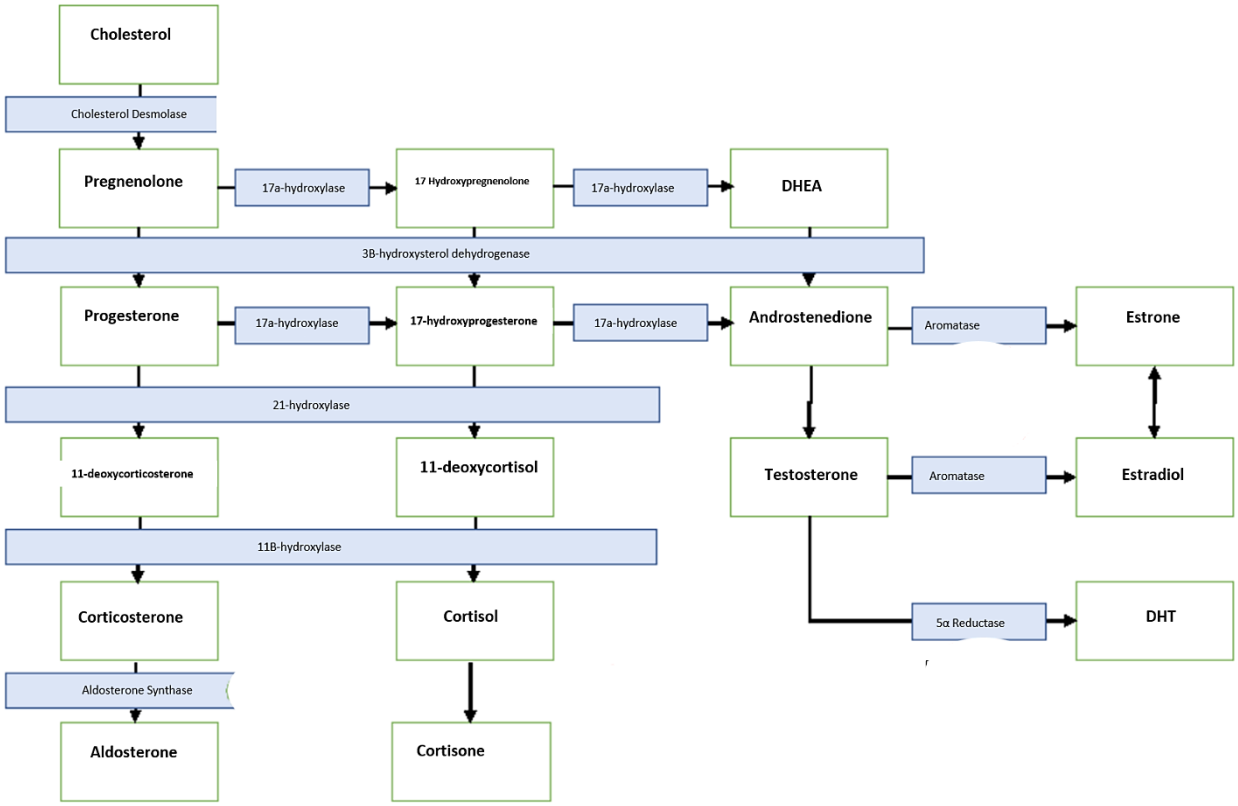


Figure 1.1

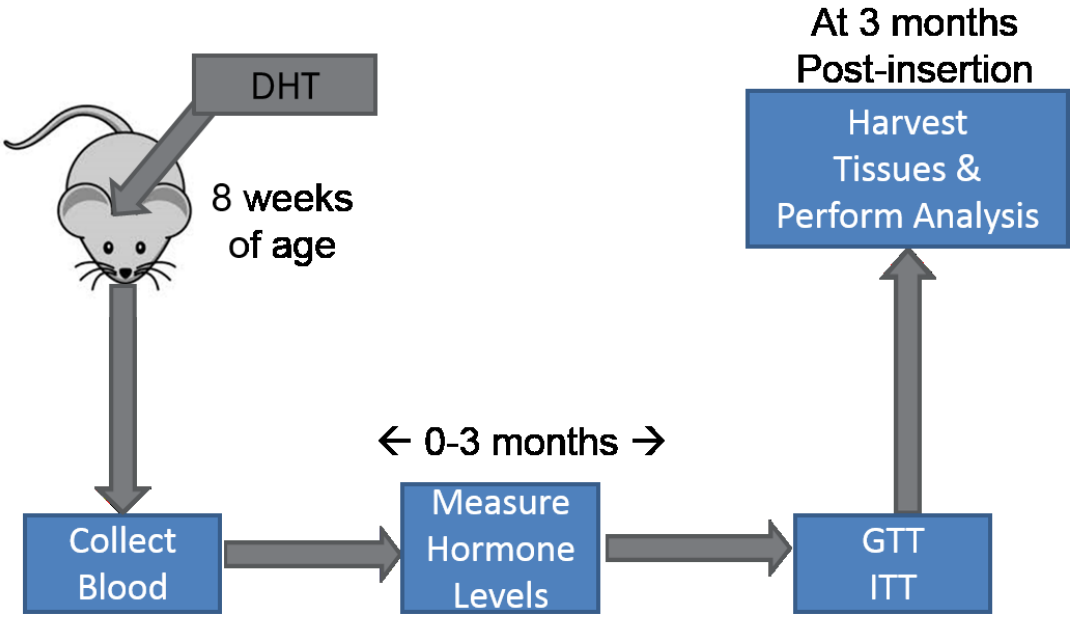
Schematic of the hypothalamic pituitary gonadal axis. Modeled is the Estrogen regulation of hypothalamic *Kiss1,* which plays an important role in mediating estrogen feedback regulation of the HPG axis.

Figure 1.2



Overview of steroidogenesis. Diagramed are the different pathways from which the sex steroids are generated from cholesterol and transformed into other steroids. Noted are the enzymes responsible for catalyzing the conversions of the steroids.

Figure 1.3



For the experimental design, 4-mm DHT pellets and 4-mm empty pellets (control) were inserted subcutaneously between the ears of the mice. The following tests were conducted over a 3-month period: Vaginal smears were evaluated for 16 days for determination of estrous cyclicity; blood was obtained weekly for the assessment of serum hormone levels, glucose, insulin, and pyruvate tolerance tests were performed to assess whole-body glucose homeostasis, pellets were replaced and reinserted every month; and at 3 months after the original insertion, tissues were collected for further analysis. MS, mass spectrometry.

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Chapter 2

A role for kisspeptin receptor in the pituitary gonadotroph in male mice

Abstract

The anterior pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) contributes to the regulation of gonadal development, gametogenesis and the secretion of the gonadal steroid hormones. The gonadotroph is primarily regulated by hypothalamic secretion of gonadotropin-releasing hormone (GnRH) from neurons of the rostral hypothalamus and is mediated by GnRH receptor signaling. Recently, kisspeptin (KISS1)/kisspeptin receptor (KISS1R) signaling in GnRH neurons has been shown by our group and others to play an essential role in hypothalamic-pituitary-gonadal (HPG) axis function. However, whether kisspeptin signaling via the KISS1R could also regulate reproductive function at the level of pituitary is not yet established. Using Cre/Lox technology, we knocked out the *Kiss1r* gene in pituitary gonadotropes (PKiRKO). Our results revealed that PKiRKO males have normal external genital development, have normal ages of puberty as assessed by preputial separation and also have comparable body and testes weight to control male mice. While there were no differences in basal serum LH and FSH levels, we observed a significant attenuation ($P < 0.05$) in GnRH stimulated luteinizing hormone (LH) levels in PKiRKO male mice compared with Control male mice. To directly assess cellular response, calcium (Ca^{2+}) assays were performed on primary pituitary cells cultured *ex vivo*, and demonstrated that pituitary cells from Control male mice were sensitive to kisspeptin, GnRH and pituitary adenylate cyclase-activating peptide (PACAP). Pituitary cells from PKiRKO male mice exhibited reduced or absent calcium responses to the aforementioned peptides. Overall, these findings indicate that the pituitary KISS1R may plan an important modulatory role in augmenting pituitary responsiveness.

Introduction

Pubertal onset is marked by an increase in the frequency and amplitude of gonadotropin-releasing hormone (GnRH) pulses by the hypothalamus, which is followed by increased secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by the anterior pituitary (Fink,2000;Pinilla L et al.,2012;Themmen & Huhtaniemi,2000; Bliss et al., 2010). Kisspeptin

and its G-protein coupled receptor (GPCR), the kisspeptin receptor (KISS1R/GPR54) have a critical role in regulating the hypothalamic-pituitary-gonadal axis in all mammalian systems (Funes et al., 2003; Messager et al., 2005; Gottsch et al., 2004; Smith et al., 2007.). Loss of function mutations of *Kiss1r* are associated with lack of puberty onset and hypogonadotropic hypogonadism in humans and rodents, whereas gain-of-function mutations produce precocious puberty in humans (Seminara et al.,2003;de Roux et al.,2003; Teles et al.,2008; Silveira et al.,2010.). In rodents, *Kiss1r* is highly expressed in hypothalamus and its role in regulating the reproductive hormone axis is firmly established (Dror et al., 2013). Conditional knock-out studies have clearly defined a role for *Kiss1r* expression in the GnRH neuron for reproductive development and fertility (Clarkson et al., 2009; Novaira et al., 2014.). Besides the hypothalamus, pituitary and gonads, *Kiss1r* is also detected in placenta, liver, pancreas and intestine where its role is less clear.

GnRH neurons located in the hypothalamus have long been thought to be the final neuroendocrine output regulating the HPG axis. This has resulted in few studies exploring the role of *Kiss1r* in the pituitary. However the ability of the pituitary to incorporate diverse cues and signals to affect reproductive output is gaining more attention. These cues and signals include gonadal hormones such as estradiol (Singh et al., 2009), testosterone (Ramaswamy et al.,2007.), progesterone (Girmus & Wise et al.,1992) and inhibin (MacLachlan et al.,1987), metabolic hormones such as insulin (Brothers et al.,2010.), and locally produced proteins such as activin (Bilezikjian et al.,2011.) Alternatively, the pituitary is accessible to be acted upon by circulating KISS1, thus activating KISS1/KISS1R signaling cascades. Both KISS1R and GnRHR are G-protein-coupled receptors (GPCRs) containing the prototypical seven transmembrane regions and can activate several GPCR mediated signaling pathways. Additionally, a number of other hypothalamic regulatory factors such as corticotropin-releasing hormone (CRH) (Hauger et al., 1993.), dopamine (Pivonello et al., 2004.), and pituitary adenylate cyclase-activating peptide (PACAP) (Harada et al., 2007.) also act via specific GPCRs.

Witham et al have demonstrated that kisspeptin increases gonadotropin gene expression in mouse primary pituitary cells in culture (Witham et al., 2013.) Additionally, it has also been shown that *Kiss1r*

expression is enhanced in the pituitary of female mice during the estradiol-induced LH surge (Clarkson et al., 2008.), suggesting a possible role for kisspeptin as part of the constellation of regulatory inputs to the pituitary required for the massive preovulatory release of gonadotropins. Our studies using a novel kisspeptin receptor knockout mouse model (PKiRKO mouse) provide evidence that kisspeptin signaling augments pituitary gonadotroph responsiveness and may play a role in regulating the male HPG axis.

Although both male and female PKiRKO mice were produced, only the male model was shown to have nearly complete knockout of *Kiss1r* in the pituitary gonadotroph and therefore we present here findings primarily from the male PKiRKO mouse. Knowledge of the precise role of kisspeptin and the kisspeptin receptor at the level of the gonadotroph will help define the complex regulation of mammalian reproduction at the level of the pituitary.

Materials and Methods

Generation of Gonadotrope-specific Kiss1R knockout mice (PKiRKO)

To generate pituitary Kiss1 receptor (*Kiss1R*) knockout (PKiRKO) mice, we crossed heterozygous floxed *Kiss1r* (fl/wt) female mice (Singh et al., 2009) with αGSU transgenic (αCre+/-) male mice (Pivonello et al., 2004; Quennell et al., 2011.). F1: female mice (*Kiss1r* fl/wt; αCre+) and male mice (*Kiss1r* fl/fl; αCre-) were crossed to produce PKiRKO mice (*Kiss1r* fl/fl; αCre+). Litter mates (*Kiss1r* fl/wt; αCre- and *Kiss1r* fl/fl; αCre-) were used as Controls. Heterozygous knock out mice (*Kiss1r* fl/wt; αCre+) mice were not included in these studies. Genotyping primers were designed to detect the presence of the floxed allele, WT allele, or knockout allele of Kiss1r: P1 sense (located in exon 1) and P3 antisense (located in exon 3). Using genomic DNA obtained from extra-pituitary organs (eg. tail), primers P1 and P3 amplify a 2096-bp amplicon (floxed *Kiss1r* allele) and 1882-bp band (WT allele) and a 1120-bp amplicon (KO allele, if the sequence between the LoxP sites is excised). Primer sequences are shown in Table S1.

Animals

Adult male and female mice (>2 months old) were used in this study. All animal studies were carried out in accordance with National Institutes of Health guidelines on animal care regulations and were approved by the Animal Care and Use Committee of the Johns Hopkins University. Mice were maintained under constant conditions of light and temperature (14: 10 h light⁄dark cycle; 22 C), and were fed a normal chow and water ad libitum.

Quantitative Real-Time PCR (qPCR)

RNA was extracted from the pituitary, liver, ovaries, uterus, testes, epididymis, adipose tissue and from two hypothalamic fragments encompassing the arcuate and the anteroventral periventricular nucleus (AVPV) as described previously. TRIzol reagent (Ambion Life Technologies, Carlsbad CA, USA) was used according to the protocol provided by the supplier. 1µg of RNA was reverse transcribed to cDNA using an iScript cDNA kit (Bio-Rad Laboratories). Real-time qPCR was performed to determine the presence and relative expression levels of *Kiss1*r mRNA in the various tissues. Real-time qPCR was performed in duplicate using SYBR Green Master Mix (Bio-Rad Laboratories) and the CFX Connect qPCR machine (Bio-Rad Laboratories). Primer sequences for the selected genes are in Table S1. For each primer set, PCR efficiency was determined by measuring a 10-fold serial dilutions of cDNA and reactions with between 95% and 105% PCR efficiency were included in subsequent analyses. Relative differences in cDNA concentration between Control and PKiRKO mice were then calculated using the comparative threshold cycle (C_t) method. In order to compare the difference of *Kiss1*r expression in the same tissue between Control and PKiRKO, a $\triangle C_t$ was calculated to normalize for internal control using the equation: C_t (Kiss1R) – C_t (18S). $\triangle\triangle C_t$ was calculated: $\triangle C_t$ (PKiRKO) - $\triangle C_t$ (WT). Relative Kiss1R mRNA levels were then calculated using the equation fold difference = $2^{-\triangle\triangle Ct}$.

Pubertal onset and assessment

Preputial separation (PPS) in males was assessed daily beginning after postnatal day 21. This consisted of determining whether the prepuce could be manually retracted with gentle pressure. PPS is testosterone dependent and thus is an indicator of activation of the reproductive axis in males. Puberty

in rodents is dependent on weight (Marty et al., 2001); hence, the weights of PKiRKO and control littermates were assessed once a week in prepuberty (day 21) through adulthood (day 49).

Hormone assays

Blood samples were collected from submandibular vein (Naik et al., 2006) between 9:00 and 10:00 AM and basal levels of serum LH and FSH were measured. The basal and stimulated (gonadotropin-releasing hormone (GnRH) stimulation test) blood were collected on different days. For the GnRH stimulation test, GnRH (catalog no. L4513–1MG; Sigma-Aldrich) was diluted with PBS to a stock solution of 583.7 ng/μL and aliquoted to individual tubes for storage at -20°C. Just before injection, stock was diluted with PBS to a final working concentration (0.02 ng/μL). GnRH was injected subcutaneously at the nape of the neck at a dose of 200 ng/kg body weight per mouse. Blood was collected 15 minutes after injection, spun at 6000g, and supernatant was stored at -20C until use. LH and FSH were measured using a Milliplex MAP immunoassay (Mouse Pituitary panel; Millipore, St Charles, Missouri) on a Luminex 200IS platform (Luminex Corporation). The assay detection limit for LH was .012 ng/mL and for FSH was .061ng/mL. The intra-assay coefficients of variation (CV) for LH and FSH were between 5 % and 9%. Serum E_2 levels were measured with a mouse/rat E_2 kit from Calbiotech (Spring Valley, CA). The sensitivity for E_2 was 3 pg/mL. Testosterone levels were measured by radioimmunoassay by the University of Virginia Ligand Assay Core (Charlottesville, Virginia).

Fertility assessment

To determine whether male PKiRKO mice were fertile relative to controls, 3 Control and 4 PKiRKO male mice were housed with proven fertile WT female mice for 14 consecutive days (1 male and 1 female per cage, total 7 cages) and then were separated. If a pregnancy ensued, one week after female gave birth, a new male was inserted. Males were rotated among the 7 cages. The duration of the fertility study was 4 months and 4 rotations for each male mouse.

Anatomy, Histology and Immunostaining

Testis

Wet testicular weights were determined in freshly dissected mice. Testes were fixed in Bouin's solution (SIGMA), and stored at 4°C. Tissues were sectioned to 5µm thickness in their entirety by the Johns Hopkins Medical Laboratories Histology Group (Baltimore, Maryland).

Pituitary

Pituitary was fixed in 4% paraformaldehyde (Affymetrix, Inc) for 2 hours on ice and immersed in 30% sucrose overnight. Pituitary was then embedded in OCT at -20°C and sectioned to 6 µm. For single labeling for KISS1R, sections of pituitary were incubated with rabbit anti-KiSS1R (Abcam) at 1:1000 dilution for 30 hours and washed with PBS overnight at 4°C. Secondary antibody (goat anti-rabbit IgG Alexa Fluor 594) was added at 1:400 dilution for 2 hours at room temperature.

For double labeling of KISS1R and LHβ sections were incubated in KISS1R- 1:3000 rabbit anti-KISS1R (Abcam); 1:2000 biotinylated goat anti-rabbit secondary antibody (vector) and avidin-biotin complex (Elite ABC kit, Vector Laboratories) and visualized with nickle/DAB chromagen. Subsequently, sections were incubated with LHβ- 1:5000 LHβ (NIDDK-AFP571292393); 1:2000 Rabbit-Anti Rat secondary antibody conjugated with Alexaflur 568 (Life technologies). Sections were imaged with an AxioCamAR camera and exported to AxioVision software.

Pituitary cell dispersion and culture

Mice (n=4 per group) were sacrificed by decapitation and pituitaries were immediately excised and placed in a 50ml conical tube containing fresh HBSS (Gibco). After brief centrifugation at 600 g for 5 minutes, the whole pituitaries were transferred to a conical tube containing fresh 10% fetal bovine serum containing DMEM. Before cell dispersion, pituitaries were washed several times with fresh DMEM. The whole pituitary was enzymatically and mechanically dispersed using 2mL of both collagenase (HBSS with 30mg/ml BSA) and pancreatin (Ca 2+/Mg2+ free HBSS with 4.5 mg/ml pancreatin) solutions. The pituitary was then further dispersed with a small magnetic stir bar for 90 minutes at 37°C and washed in Ca 2+/Mg2+ free HBSS before being transferred into a 15 ml centrifuge tube containing fresh 10% FBS DMEM. Cell viability, was always higher than 90% as estimated by the trypan blue exclusion test in a Neubauer chamber. Before plating cells, Matrigel (Corning-

Tewksbury, MA) was used to coat the surface of the cell culture plates for 2 hours. For LH and FSH in vitro secretion verification, pituitary cells were plated at a density of $1x10^6$ cells per well of a six-well dish. For the calcium flux assay, cells were plated at a density of ~15,000 cells per well in 384 well plate. After allowing the cells to attach for 24 hours in an incubator (37°C and 5% CO2), the cell culture media was then changed to media containing KP-10 (EMD Biosciences, Inc., San Diego, CA, USA) and different doses of GnRH and/or PACAP -38 (Pituitary Adenylate Cyclase Activating Peptide-38, BACHEM- Bubendorf, Switzerland). To validate the health and function of the cells, a GnRH time course study was performed on primary pituitary cells harvested from Control male mice. Groups of cells were treated with 30nM GnRH, for a duration of 30, 60 and 360 (6 hours) minutes. A significant increase in media LH levels was observed in mice pituitary cells when treated 30nM GnRH confirming the viability of the cells (Figure 2.18).

Calcium Flux Assay

Male primary pituitary cells were plated in 10% FBS DMEM at a density of ~ 15,000 cells (100ul/well) overnight in a Matrigel coated 384 well plate. Cells were loaded with the dye fluo-4 (Ex/Em-480/540) by adding 1X dye-loading solution (BD Calcium Assay kit (BD Biosciences, Rockville, MD) and incubating for 1 hour at 37°C. GnRH, KP10 and/or PACAP-38 were added by an FDSS (Hamamatsu) machine and the data was recorded simultaneously. The kinetics of intracellular calcium flux were calculated using the fluo-4 channels from the FDSS machine. Fluo-4 is nonfluorescent when not bound to Ca^{2+}. A kinetic curve of calcium response to different concentrations of agonists was produced.

Data Analysis and Statistics

All data are reported as means ± SEM, with 'n' representing the number of mice or samples used in each of the experimental groups. Data were analyzed by unpaired t tests or a one-way ANOVA with a Bonferroni post-test used for 3 or more groups using Prism software (GraphPad Software, Inc, La Jolla, CA). The magnitude of intracellular calcium flux was graphed, and the changes in this magnitude of

fluorescent intensity upon agonist addition was then plotted with Prism software. For all calcium assay kinetic curves, maximal 480/540 ratios known as peak intensity were graphed as box plots.

Results

Generation of pituitary specific *Kiss1r* knockout mice

PKiRKO (Kiss1R$^{fl/fl}$;αCre^{+}) mice were generated by Cre recombinase mediated excision of exon 2 of the *Kiss1r*, resulting in loss of function of the *Kiss1r* gene in pituitary, as shown in the schematic diagram of Figure 2.1A. Representative PCR genotyping is displayed in Figure 2.1 B (for *Kiss1r* alleles) and C (for αCre transgene).

Pituitary and tissue *Kiss1r* mRNA expression in PKiRKO mice

Disruption of *Kiss1r* expression was assessed by real-time quantitative PCR (RT-qPCR) and immunostaining. RT-qPCR demonstrated a reduction in *Kiss1r* mRNA by 88% (Figure 2.2 A) and 64% (Figure 2.2 B) in the pituitary of male and female PKiRKO mice (n=8), respectively, compared with Control mice (n=8). In contrast, no change in *Kiss1r* mRNA was observed in other tissues including the hypothalamus, adipose tissue, liver, or gonads ($P > 0.05$, n =4) (Figure 2.2 A and B). Immunohistochemistry revealed KISS1R (black signal) presence in gonadotrope cells of control pituitary (Figure 2.2 B, E). Reduced levels of KISS1R protein in PKiRKO mice paralleled the mRNA levels as shown in (Figure 2.2 C, F). As shown in the immunostaining, a more robust knockout of *Kiss1r* was observed in the male mice than in the female mice. Due to the incomplete knockout of *Kiss1r* in females, we continued our study focused on the male Control and PKiRKO mice. Female analysis of puberty, fertility, gonadotrope levels, ovarian morphology were carried out in parallel (Figure 2.11-. Double labeling of LH receptor (LHR) and KISS1R revealed that KISS1R protein levels were nearly absent in the pituitary gonadotrophs of PKiRKO males (Figure 2.17).

PKiRKO mice showed no external morphologic abnormalities

Reproductive development and function of the male PKiRKO mice was analyzed. External anatomic abnormalities were not observed in PKiRKO mice compared with that of littermate Controls

(Figure 2.8 A and B). PKiRKO males did not exhibit microphallus or decreased anogenital distance compared to Controls. (Figure 2.8).

Pubertal onset and body/gonad weights are normal in PKiRKO mice

In males, PPS (preputial separation) is an androgen dependent process that serves as an external sign of male puberty onset(Marty et al., 2001). In males, no significant difference was observed in age of PPS between the Control and PKiRKO groups (Figure 2.4A). Because body weight has been demonstrated to be highly associated with age of puberty(Castellano et al., 2006), we measured the weight of the mice throughout the puberty assessment (Figure 2.3A) and found no difference in body weight (Figure 2.3A) and testes weight (Figure 2.3B) between Control and PKiRKO mice. Furthermore the numbers of sperm in the seminiferous tubules was not statistically different between Control and PKiRKO male mice (Figure 2.11 A).

No impaired fertility in PKiRKO mice

Fertility was further examined in Control and PKiRKO mice using a rotating mating protocol. PKiRKO male mice demonstrated a normal ability to produce offspring, as shown in Figure 2.4 B-E. Female control mice had the same number of litters with PKiRKO and Control sires (Figure 2.4 C) and a similar number of pups per litter (Figure 2.4 E). Female control mice bore their first litter with a similar latency after introduction to PKiRKO males or Control males (Figure 2.4 D).

PKiRKO mice have normal basal serum LH and FSH levels

Morning serum samples were obtained from non-breeding, post pubertal mice and serum LH and FSH hormone levels were determined. Basal serum LH and FSH values in PKiRKO mice were not significantly different in PKiRKO males relative to Control mice (Figure 2.5A and B). Although not significantly different, there was a trend for lower basal LH and FSH levels in male PKiRKO male mice than Control male mice.

PKiRKO male mice exhibit decreased GnRH responsiveness by the pituitary gonadotrophs

To further assess pituitary function, GnRH-stimulation tests were performed, and LH levels assessed in blood harvested after 10 minutes. Serum LH values in PKiRKO and Control mice were

significantly increased after GnRH stimulation compared with the baseline levels of Control mice (Figure 2.5A). While basal gonadotropin levels were not different, importantly, there was a significant attenuation in the GnRH-stimulated LH serum in male PKiRKO mice when compared to their control littermates. There were no significant changes in FSH levels in both Control and PKiRKO males before and after GnRH stimulation. (Figure 2.5 B). To understand the mechanism contributing to altered pituitary sensitivity, we measured *Gnrhr* and *Esr1 (*Estrogen receptor alpha*)* in pituitary of control and PKiRKO mice. Both *Gnrhr* (Figure 6A) and *Esr1* (Figure 2.6 B) mRNA were decreased in PKiRKO mice compared with Control mice.

PKIRKO male primary pituitary cells show differential calcium response *ex vitro*

To directly study the effects of disrupted KISS1R signaling on the pituitary gonadotroph, we used an *ex vivo* primary culture system to assess calcium flux dynamics of male Control and PKiRKO mice pituitary cells. Over a period of 15 minutes (900 secs) we measured relative Ca^{2+} levels, including maximum Ca^{2+} amplitude, as assessed by 480/450 ratios of fluo-4 (Kinetic data; Figures 2.7 A-F, Peak amplitude; Figures 2.7 G-L). Following a one minute recording of baseline calcium both Control and PKiRKO primary pituitary cells were treated with or without KP10 (10nM). When treated with kisspeptin (10 nM), calcium levels increased significantly in Control cells (Figure 2.7 C, I), while no increase in intracellular calcium was observed following KP-10 treatment in the PKiRKO cells (Figure 2.7 D, J).

GnRH, acting through the GnRHR, a GPCR, regulates pituitary expression and secretion of the gonadotropins. Cells were treated with varying doses of GnRH -10, 30 &100 nM as well as a combination treatment of 1nM KP10 & 30nM GnRH. As shown in Figure 2.7 A and further quantified in Figure 2.7 G, all doses of GnRH caused increased intracellular calcium in Control male cells but not in pituitary cells from PKiRKO mice. Co-treatment of 30nM GnRH with 1nM KP10 augmented the response relative to 30nM GnRH alone, but only in Control cells.

PACAP is a peptide that has been shown to directly regulate pituitary gonadotroph function causing increases in LH expression. Pituitary cells were exposed to PACAP-38 at doses of 10, 50 and

100 nM for 900 seconds. Figure 2.7 E and F shows traces of Control and PKiRKO pituitary cells respectively. PACAP-38 treatment elicited intracellular calcium increases in Control (Figure 2.7 E) but not in PKiRKO male primary pituitary cell cultures (Figure 2.7 F). This difference is quantified in Figures 2.7 K and L where there was no significant difference in maximum amplitude of the Ca^{2+} signal between the PACAP treated and untreated wells.

Discussion

While kisspeptin was originally discovered by cancer researchers, its most well-established role is as a potent activator of the GnRH neuron, and thus as a critical regulator of hypothalamic control of reproduction. The role of the KISS1/KISS1R signaling system has widened as investigators have identified extra-hypothalamic roles in tissues such as the liver, uterus, testes and ovary (Gutierrez-Pascual et al., 2007.). In addition, expression of KISS1R in the pituitary gonadotroph (Shacham et al., 2001) points to the pituitary as a possible target for kisspeptin action. The functional significance of pituitary KISS1R is not well defined. To directly evaluate the role of pituitary kisspeptin signaling, we developed the PKiRKO mouse model lacking KISS1R in the pituitary gonadotroph. We demonstrate that KISS1 signaling at the level of the pituitary may serve a role in modulating GnRH stimulated gonadotropin secretion.

In humans and most mammals hypothalamic GnRH secretion drives the synthesis and secretion of the gonadotropic hormones LH and FSH from the secretory cells of the anterior pituitary. GnRH binding to the GnRHR at the surface of the gonadotropes activates intracellular signaling pathways triggering membrane depolarization and a rapid change in intracellular Ca^{2+} concentration, eliciting secretion of LH and FSH (Shacham et al., 2001). The pituitary, by virtue of its location outside of the blood brain barrier, is poised to serve as an integrative sensor for peripheral metabolic status, peripheral reproductive status and central signals regulating reproduction.

While GnRH plays a dominant role in regulating gonadotroph secretion, gonadal hormones and metabolic hormonal signals such as insulin may also contribute to regulation of pituitary function.

Kisspeptin's growing recognition as having extra- hypothalamic actions, led us to explore whether kisspeptin impacts pituitary and gonadotroph function directly. Ian Clarke's group detected KISS1 in the hypophyseal portal circulation of ewes, presumably of hypothalamic origin, and further noted a role for KISS1 in stimulating pituitary LH secretion (Smith et al., 2008). However, they noted that the concentration in the portal circulation was too low to exert effects at the pituitary. Pituitary KISS1R could be a target of either centrally or peripherally derived KISS1. The source of kisspeptin could be hypothalamic or from peripheral tissues such as liver (Song et al., 2014) or gonad (Salehi et al., 2015) where kisspeptin could be secreted into the circulation and access anterior pituitary cells.

Investigators have observed KISS1 induction of LH secretion in cultured primary rat and primate pituitary cells(Navarro et al., 2005; Luque et al., 2011) and up-regulation of gonadotropin gene β-subunits, LHβ and FSHβ gene expression in LβT cells(Witham et al., 2013.), although others have seen no direct effect of KISS1 on LH or FSH secretion(Matsui et al., 2004; Thompson et al., 2004.). A number of studies have suggested that KISS1 may act directly on pituitary gonadotropes to stimulate LH release (Gutierrez-Pascual et al., 2007; Luque et al., 2011; Castellano et al., 2006.) or to increase gonadotropin or *Gnrhr* gene expression (Naor et al., 1980). We found that KISS1R is expressed on mouse pituitary gonadotrophs (Figure 2.2 and 2.17) which supports studies in rat (Richard et al., 2008) and sheep (Backholer et al., 2009) demonstrating co-expression of LH and KISS1R in the pituitary.

Global *Kiss1r* knockout mice have demonstrated KISS1R is vital for normal pubertal onset and estrous cyclicity (Messager et al., 2005; d'Anglemont et al., 2009; Lapatto et al., 2007). This phenotype was phenocopied in GnRH neuron-specific Kiss1RKO mice (Novaira et al., 2014) confirming an essential role for kisspeptin signaling at the level of the GnRH neuron. These studies however do not preclude a role for kisspeptin signaling at other levels of the axis including at the level of the pituitary. We did not identify a phenotype in female PKiRKO mice, but due to an inadequate knock-out of the gene in the female pituitary (Figure 2.2 D), we are not equipped to conclude the receptor plays no role in the female. Phenotyping of the male PKiRKO mice revealed normal pubertal onset (Figure 2.4),

normal genital anatomy (Figure 2.8), as well as normal fertility (Figure 2.4). It is possible that a complete KO of the receptor in the male would result in changes in neuroendocrine regulation of puberty and/or fertility.

While these physiological parameters were normal, direct assessment of pituitary function demonstrates there is a modulatory role for the KISS1/KISS1R signaling system in the pituitary gonadotroph. To directly probe pituitary function, a GnRH stimulation test was administered. We observed that exogenous GnRH administered to PKiRKO mice was able to elicit increases in basal serum LH levels, however, the magnitude of stimulated secretion was significantly attenuated compared to Controls (Figure 2.5A). This was not associated with differences in basal gonadotropin levels or peripheral serum testosterone between PKiRKO and Control male mice (Figure 2.13) indicating the hypothalamic homeostatic set point was not impacted, despite the modulated function of the pituitary.

Our data suggest the decrease in stimulated LH secretion in male PKiRKO mice pituitaries is due to the reduced expression of *Gnrhr* mRNA levels - significantly reduced by up to 40% as determined by qPCR (Figure 2.6 A). This finding provides evidence of possible kisspeptin and GnRH signaling crosstalk. This crosstalk is not surprising as both KISS1R and GnRHR are typical GPCRs having relatively similar intracellular mechanism of action. KISS1R and GnRHR are stimulated upon activation of the homologous heterotrimeric G proteins by a ligand such as an ion, peptide or lipid (Kobilka, 2007). This then leads to further activation of downstream signaling pathways (e.g. MAPK, PLC, PKC) resulting in cellular responses. Control of *Gnrhr* expression in the gonadotropes is markedly regulated by GnRH itself (Nathwani et al.,2000) with pulsatile stimulation of the GnRHR leading to increased concentrations of the receptor, while continuous exposure to GnRH downregulating *Gnrhr* mRNA and gonadotropin secretion(Belchetz et al., 1978). The Clay group demonstrated convincingly that GnRH regulation of the *Gnrhr* gene is dependent on ERK activation of promoter elements in the *Gnrhr* gene (Brett et al., 1999). Perhaps kisspeptin signaling via KISS1R can modulate the ERK

pathway providing a mechanism for crosstalk between KISS1R and GnRHR. Further studies will need to be performed to assess the mechanism for KISS1R regulation of *Gnrhr* expression.

Gonadal steroid feedback regulation of the pituitary gonadotroph is mediated in large part ERalpha. In both males and females pituitary gonadotroph ESR1 is another important determinant of the function of the reproductive endocrine gland (Gieske et al., 2008). An interesting study done in male mice by Lindzey et al (Lindzey et al., 1998), showed that *Esr1* knockout mice had FSH levels that were 20% higher than those of WT males. Furthermore LHbeta mRNA and serum LH levels in non-castrated male ESR1KO mice were significantly higher than those of their WT male counterparts. These findings conclude that ESR1 is important for normal physiology of male gonadotrophs. As a nuclear hormone receptor, ESR1 affects expression of many downstream target genes including the gonadotropin subunits LHβ, FSH β and common alpha-glycoprotein (CGA) (Ferris & Shupnik, 2006). We measured *Esr1* mRNA levels using QPCR and showed there was significantly decreased expression in male PKiRKO mice versus Control (Figure 2.6 B). We propose that reduced KISS1R signaling in the pituitary causes a reduction in *Gnrhr* expression, and coupled with reduced ESR1 action, leads to a reduction in LH secretion after GnRH stimulation in PKiRKO males. A link between pituitary ESR1 and GnRHR was shown in a study of female mice which found that *Esr1* potentiated LH hormone release on subsequent GnRH stimulations (Kim et al.,2011) by increasing expression of GnRHR in gonadotrophs (Strauss et al., 2018). This mechanism could be similar to our findings of decreased GnRH induced LH secretion in the PKiRKO model.

Calcium is an essential component of the secretory response in pituitary gonadotrophs. Changes in intracellular calcium are measured to ascertain overall cell function/activity. Using primary cultured pituitary cells, we measured intracellular calcium response to the hypophysiotropic hormones GnRH, KP-10, and PACAP-38. Liu et al (Liu et al., 2008) performed one of the few studies to directly assess the effect of kisspeptin signaling on calcium, using real-time calcium imaging. They found kisspeptin

caused an approximately 10% increase in intracellular calcium levels in GnRH neurons mediated by activation of phospholipase C.

Treating primary pituitaries with exogenous kisspeptin (10nM of KP-10) stimulated calcium increases in Control cells (Figure 2.7 A, G). However, in PKiRKO cells 10nM KP-10 was unable to stimulate an increase in calcium levels (Figure 2.7 B, H) confirming the effects of KP-10 were being mediated via the KISS1R. Primary pituitary cells from Control mice were responsive to GnRH and exhibited increased intracellular calcium levels in response to increasing doses of GnRH (Figure 2.7 C,I). In contrast, PKiRKO primary pituitary cells failed to respond to 10, 30 or even 100 nM of GnRH (Figure 2.7 D) as quantified by maximal Ca^{2+} signal in Figure 2.7J. *In situ*, the pituitary gland is bathed in microvasculature by the hypophyseal portal system. This system of blood vessels links not only the hypothalamus with the anterior pituitary, but also delivers peripherally derived hormones and peptides(Lubahn et al., 1993). It is likely that both kisspeptin and GnRH are concurrently activating their receptors on the pituitary. Thus, co-treatment of pituitary cells with both KP-10 and GnRH was also tested. Both PKiRKO and Control primary pituitary cells were treated with 30nm GnRH and 1nM KP-10. As shown in Figure 2.7 C & I, co-treatment was able to potentiate intracellular calcium increase in Control pituitaries. This potentiation of intracellular calcium release after co-treatment was absent in PKiRKO mice (Figure 2.7 D, J).

PACAP-38, before being placed in the VIP family of peptides, was shown to be a strong stimulator of pituitary adenylate cyclase leading to increased accumulation of cAMP and in turn downstream cellular responses(Miyata et al., 1989). Although still poorly understood, the link between KISS1/KISS1R and PACAP signaling has recently gained more attention. Anatomically, neurons expressing PACAP in the ventral premamillary nucleus of female mice converged with kisspeptin neurons of the arcuate and AVPV nuclei. Cre-Lox deletion of PACAP from the ventral premamillary resulted in delayed puberty onset and impaired reproductive function in female, but not male mice(Ross et al., 2018). Further evidence for kisspeptin and PACAP signaling crosstalk was provided by Mijiddorj

and colleagues using mouse LbetaT cells showed that KP-10 treatment led to increases in PACAP receptor expression.(Mijiddorj et al., 2017) Both KISS1R and PACAP receptor (PAC1, VPAC1, or VPAC2) are GPCR'S(Schytz et al., 2010)] which respond to ligands by activation of adenylyl cyclase (AC) in order to increase intracellular cAMP levels. It is known that AC is regulated by G proteins (Gs stimulating activity and Gi inhibiting it), thus kisspeptin and PACAP crosstalk could be mediated through the AC pathway, but we did not measure AC levels in this study.

Our findings show that pituitary cells from Control mice exhibited increased intracellular calcium in response to PACAP-38 (Figure 2.7 E, K). In contrast, the response of PKiRKO primary cells was attenuated at all three different doses of PACAP-38, with no significant increase in Ca^{2+} in PKiRKO cells in response to PACAP-38 treatment (Figure 2.7 F, L). This is similar to the findings for GnRH, - a dramatic reduction in response- suggesting common intracellular signaling pathways may be impacted due to lack of KISS1R. Therefore, KISS1R may be integral to the ability of the pituitary to fully respond to both GnRH and PACAP signaling. These calcium assay findings suggest KISS1/KISS1R signaling in the pituitary helps to set a tone for the activity of the gland, with the absence of KISS1R resulting in reduced sensitivity and responsivity of the pituitary to KP10, GnRH/PACAP. It is possible that multiple phosphorylation cascades, cAMP levels and secondary messengers (Hauser et al., 2017) controlled by GPCR's are affected in PKiRKO male mice. Whether responsiveness to other pituitary-GPCR mediated signals, such as some common small-molecule neurotransmitters including monoamines such as serotonin, dopamine, norepinephrine (Seasholtz et al., 2009), are impacted remains to be found. This finding demonstrates the importance of KISS1R to the overall functionality of the pituitary.

In summary, the results of this study illustrate that disruption of kisspeptin signaling in gonadotrophs could play an important modulatory role in regulating pituitary function. Although we did not document any reproductive or metabolic defects in the PKiRKO male mice, further investigation into the pituitary function of the mice revealed that that deletion of *Kiss1r* in the pituitary compromises pituitary function

in male mice. This reduction in pituitary function was exhibited as male PKiRKO mice had a reduced responsiveness to GnRH stimulation possibly due to reduced transcriptional regulation of the pituitary genes *Esr1* and *Gnrhr*. Direct study of the male PKiRKO pituitary *ex vivo* additionally revealed that the effects of hypophysiotropic hormones on pituitary function were impacted by loss of *Kiss1r.* KP-10 (no effect), GnRH and PACAP-38 (reduced responsiveness) all had differential effects on the pituitary cells from PKiRKO mice when compared to those from Control male mice. The lack of an observed phenotype in the female PKiRKO mice is not considered conclusive. We may have observed a phenotype in females if a more complete KO could have been achieved, and similarly, the phenotype in males might have been more pronounced if a more complete KO was obtained. Our studies provide a rationale for developing conditional KO models using mouse lines with higher levels of CRE expression in the pituitary gonadotroph, such as the Fshb-iCre (Wang et al., 2016) or GnRHR-CRE mice(Wen et al., 2008). Overall this study reveals a potentially important modulatory effect of KISS1/KISS1R signaling in the pituitary in male mice that could identify a locus of action for peripherally derived KISS1 in the regulation of reproductive function

Acknowledgments

I would like to thank Yaping Ma for her contributions to the project. I would like to express my profound gratitude to the staff of the Johns Hopkins Research Animal Facility and Johns Hopkins Medical Laboratories Histology Group.

Table 2.1. Primer sequences used for genotyping and for qRT-PCR.

Gene name	**Forward primer**	**Reverse primer**
floxed allele, WT allele, or knockout allele of *Kiss1r*	P1 sense (located in exon 1) CTGGTCGGAAACTCATTGGT	P3 antisense (located in exon 1) AGAGTGGCACATGTGGCTTG
αCre^{+} transgene	GCCACCACCGCCCTGCTTAAGTAA	CATCTTCAGGTTCTGCGGGAAAC
QPCR Primers	**Forward primer**	**Reverse primer**
Kiss1r	CTGCCACAGACGTCACTTTC	ACATACCAGCGGTCCACACT
GnRHR	CAGCTTTCATGATGGTGGTG	TAGCGAATGCGACTGTCATC
ERα	CGCCTAGCTCAGCTCCTTCT	GATGCTCCATGCCTTTGTTAC
18s	GCATGGCCGTTCTTAGTTGG	TGCCAGAGTCTCGTTCGTTA

Figures

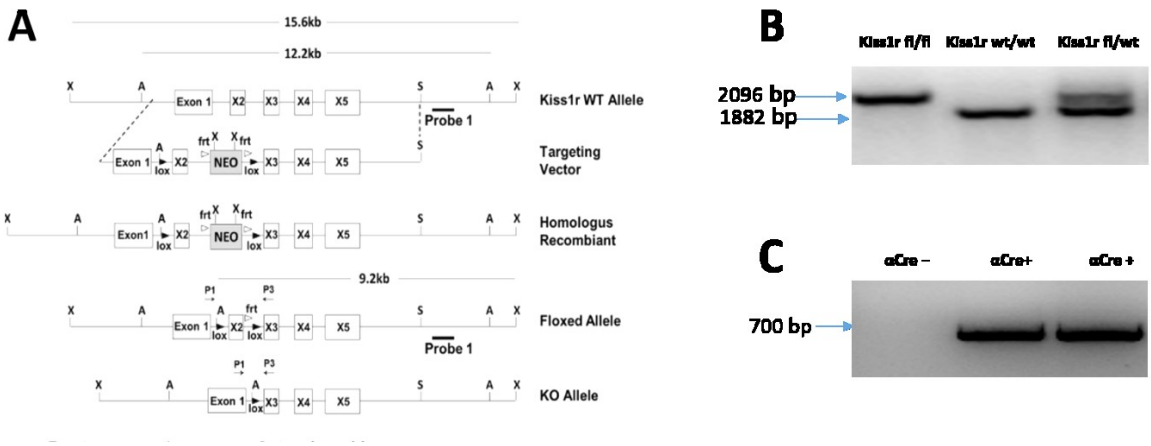
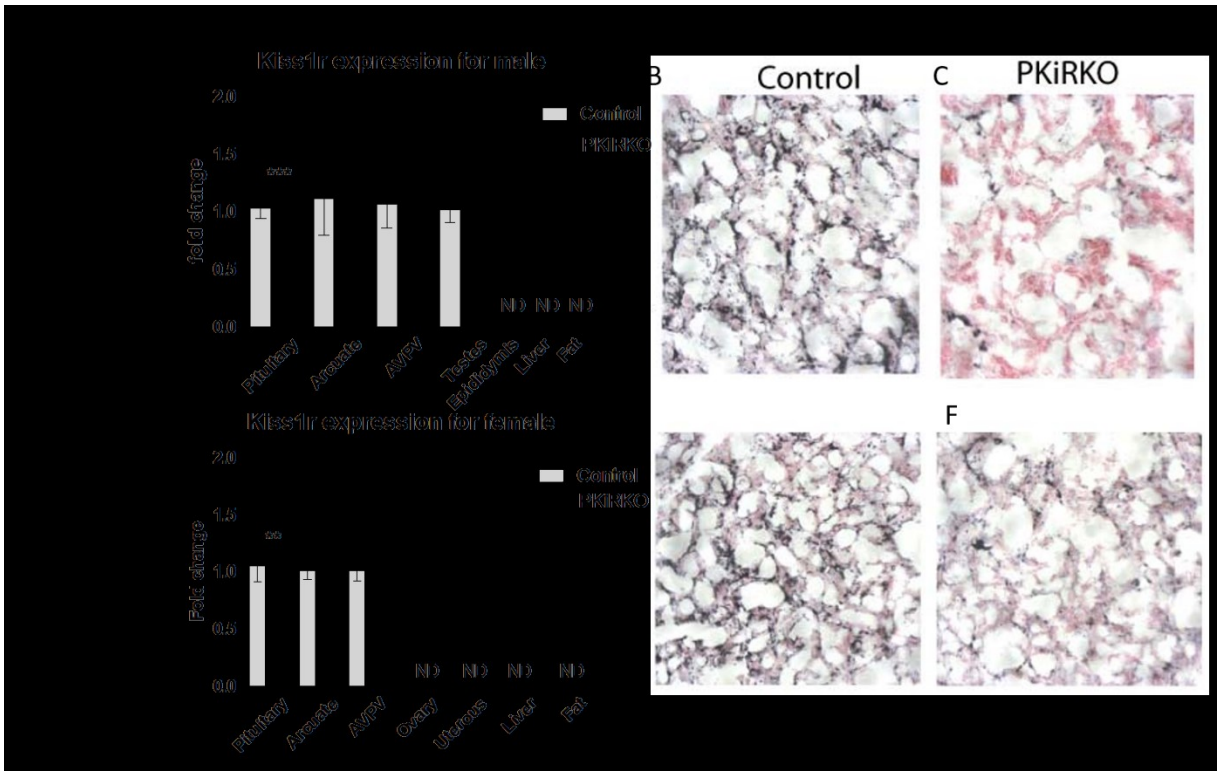


Figure 2.1 Development of the PKiRKO mouse.

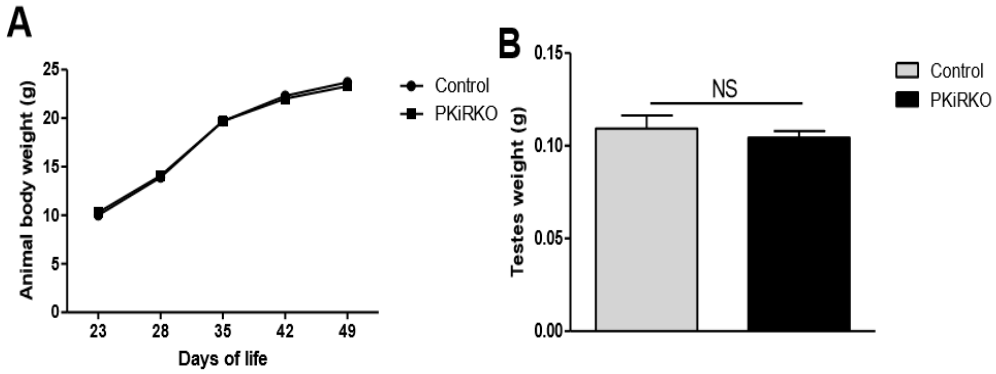
(A) Schematic diagrams of constructs used to generate PKiRKO mice. Mice bearing LoxP sites flanking exon 2 of the Kiss1r were crossed with transgenic mice expressing Cre recombinase specifically in Gonadotrophs (αGSU). (B) Genotyping by PCR analysis of the genomic DNA produced a 2096 bp amplicon in mice with a floxed allele and an 1882 bp amplicon in WT mice, also shown are both bands present in the heterozygous floxed-*Kiss1r*. (C) Lane 1-αCre^{+} transgene negative. Lane 2 & 3- αCre^{+} transgene positive mice. Amplicon size is 700 bp

Figure 2.2 Pituitary specific disruption of Kisspeptin Receptor.



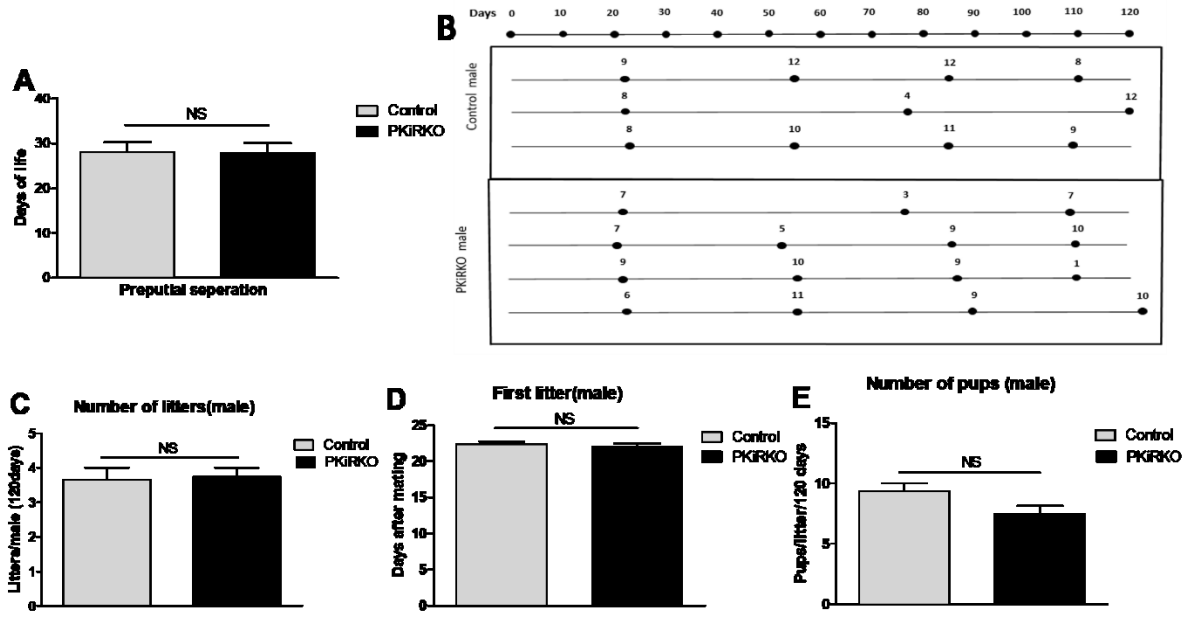
Kiss1r is reduced in the pituitary of PKiRKO mice. Quantitative RT-PCR analysis of Kiss1r mRNA extracted from male (A) and female (D) mouse tissues. *Kiss1r* was significantly reduced (87.6% for male, 63.7% for female) in the pituitary of PKiRKO (KO) mice compared with that in wide type (WT) mice, but no difference in *Kiss1r* expression was observed in other tissues. Data are means±SEM relative to the control group as determined by a two tailed Students T-test. (n=8 for pituitary, n=4 for other tissues). WT, wild-type; AVPV, anteroventral periventricular nucleus; ND, not detected; **P≤0.01;***P≤0.001. Immunostaining of *Kiss1r,* showing *Kiss1r* localized to the cytomembrane in the WT mice pituitary (B & E). Red fluorescence (*Kiss1r*) is expressed in the cytomembrane, and blue fluorescence is nuclear 4, 6-diamidino-2-phenylindole (DAPI).

Figure 2.3. No Differences in Body or Gonad Weight



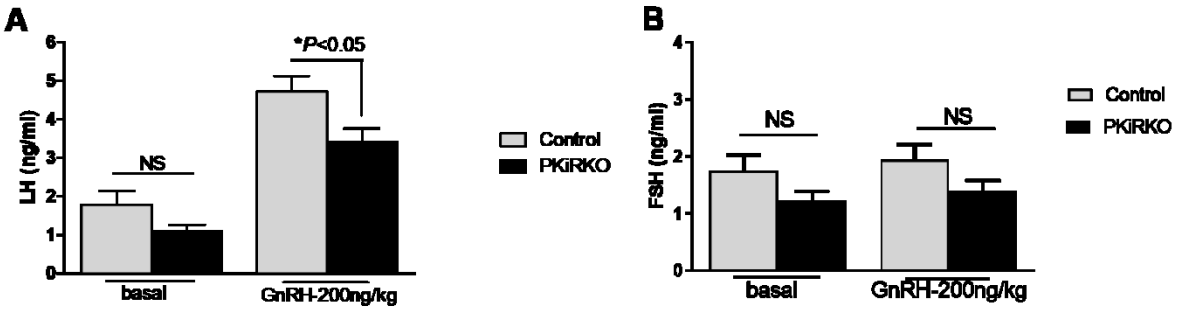
(A) Body weight change over time was not different between PKiRKO (n=15) and control male (n=15). (B) The weight of testes were not significantly different between PKiRKO and control male mice (n=6). Values are mean ± SEM relative to the control group as determined by a two tailed Students T-test. NS, not significant.

Figure 2.4. Male PKiRKO Mice Have Normal Fertility.



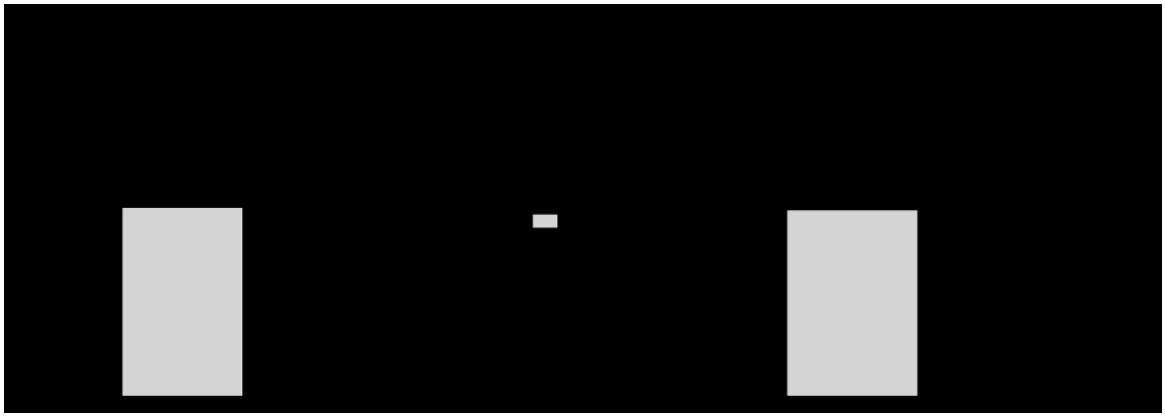
(A) No significant difference was observed in age of preputial separation (PPS) in males. (28.1 ± 2.2 [control] vs 27.8 ±2.3 [PKiRKO]) control (n=15) PKiRKO (n=15). (B) Male mice were mated with WT female mice for 4 rounds (total 120 days). Each line represents an individual male mouse. The black dot represents the day that each litter was born after introduction to female. Number on the top of the line represents how many pups in each litter. Top panel is male control mice. Bottom panel is PKiRKO male mice. (C) Total numbers of litters per male was not significantly different between control and PKiRKO mice during the 120 days. (D) After introduction with WT female, the day of first litter was recorded in both groups. (E) Number of pups per litter was also not significantly different between control and PKiRKO mice. Values are mean ± SEM relative to the control group as determined by a two tailed Students T-test. NS, not significant.

Figure 2.5. Pituitary Hormone Levels.



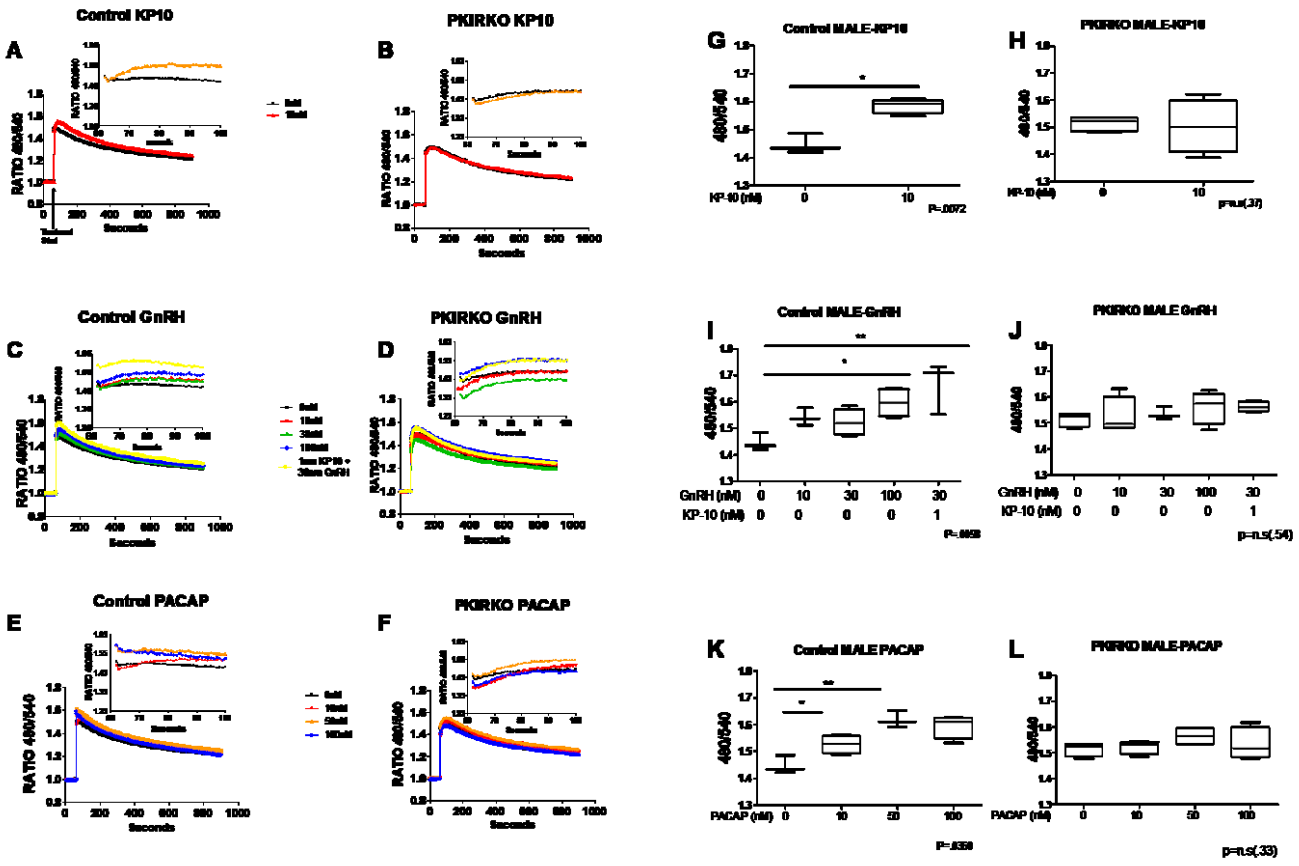
Pituitary response to GnRH stimulation was assessed in PKiRKO mice. Basal and Stimulated Serum LH (A) and FSH (B) were significantly increased in both control and PKiRKO groups following GnRH (200ng/kg) administration (n=6). GnRH stimulated LH was lower in PKiRKO than that in control male mice (n=6). (Basal LH=PKiRKO male: 1.08±0.44ng/mL, n=6 and control male: 1.79±0.86 ng/mL, n=6, P＞0.05) (Basal FSH=PKiRKO: 1.22±0.42 ng/mL, n=6 and control: 1.75±0.65 ng/mL, n=6) (GnRH Stimulated LH PKiRKO male: 3.40 ± 0.86 ng/mL, P=0.0002, n = 6 and GnRH Stimulated LH control male: 4.71±1.01 ng/mL, P=0.0003, n=6)Values are means±SEM relative to the control group as determined by a two tailed Students T-test. NS, not significant. .*P≤0.05;**P≤0.01;***P≤0.001. NS, no significance.

Figure 2.6. Receptor Expression in the Pituitary.



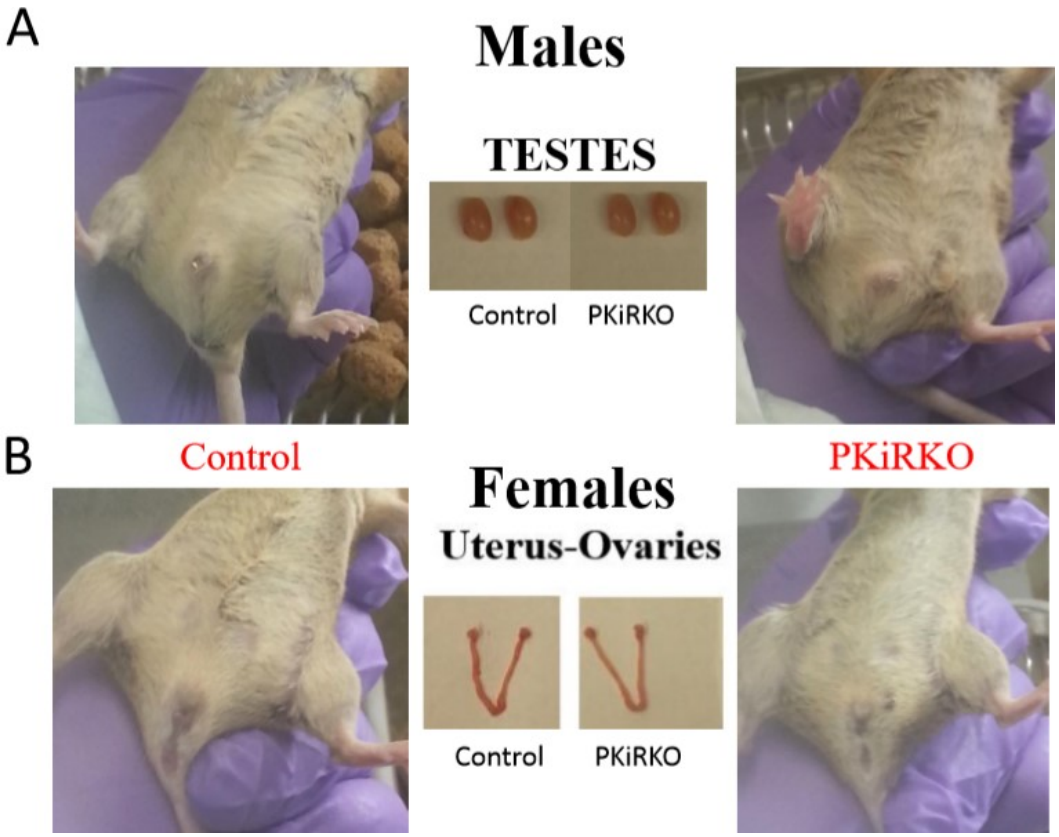
(A) GnRH receptor (*Gnrhr*) mRNA was significantly lower in PKiRKO male mice (n=8) than control male mice (n=8). (GnRHR; PKiRKO: 0.74±0.21, n=8 and control: 1.04 ±0.32, n=8, $P < 0.05$) (ER alpha; PKiRKO: 0.79±0.19, n=8 and control: 1.01±0.18 ng/mL, n=8. Values are mean ± SEM $P < 0.05$ relative to the control group as determined by a two tailed Students T-test.

Figure 2.7. Calcium Assay of Primary Pituitaries.



(A-F) Representative traces of calcium responses to agonist. A baseline measure of calcium (Ratio 480/540) was recorded for 60 seconds before the addition of agonist. Calcium flux data was then recorded for an additional 840 seconds for a total observation time of 900 seconds. (Inset) Graph showing a "zoomed" in version of the traces, highlighting calcium dynamics 60-100 seconds after addition of the hormone. (G-L) Box plots of maximal Ca^{2+} 450/540 ratios from representative traces. Primary pituitary cells were treated with one dose of KP-10(10nM) –A,B,G,H, varying doses of GnRH (10, 30 and 100 nM)-C,D,I,J and varying doses of PACAP 38 (10, 50 and 100nM)-E,F,K,L.Values are means ±SEM *P≤0.05;**P≤0.01 as determined by a two tailed Students T-test or one way ANOVA followed by Tukey's *post-hoc* test.

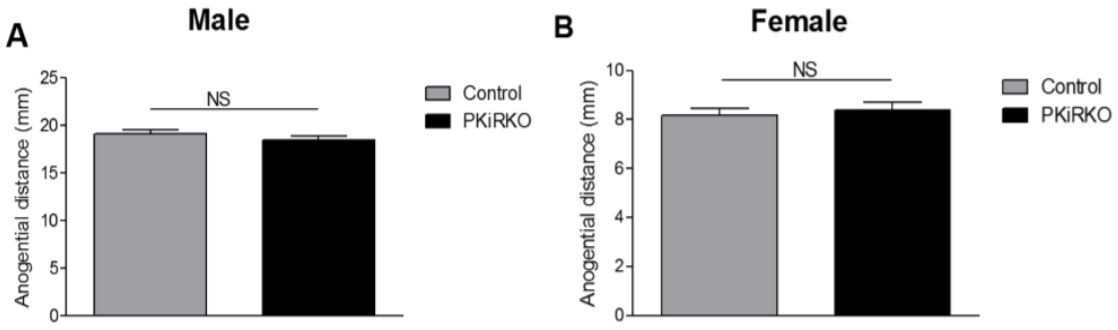
Figure 2.8



External anatomy and puberty assessment of (A) Males (B) Females.

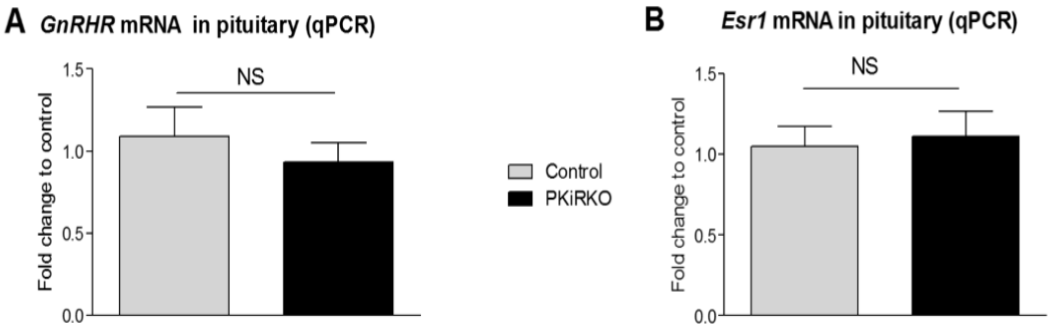
External anatomic abnormalities were not observed in PKiRKO male and female mice.

Figure 2.9



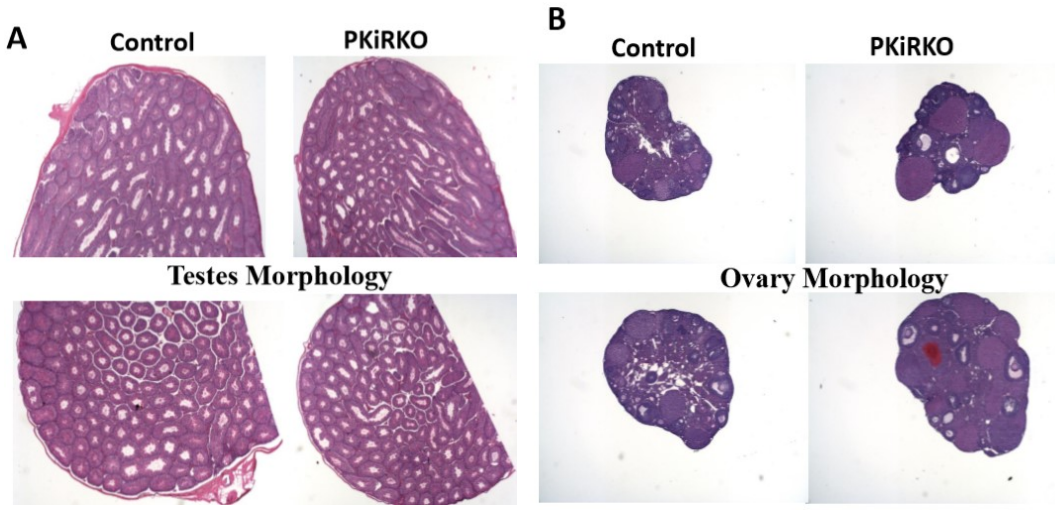
PKiRKO (A) Males and (B) Females exhibited no difference in anogenital distance compared to controls, and no obvious differences in gonadal physiology were seen. Values are means ±SEM as determined by a two tailed Students T-test NS, no significance.

Figure 2.10



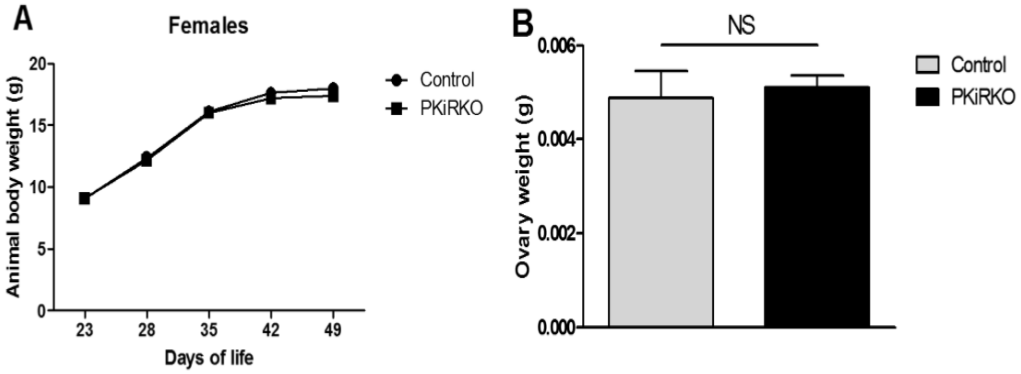
(A) GnRHR mRNA was not significantly different between control and PKiRKO male mice (n=8). (B) Esr1 mRNA levels were also not significantly different between control and PKiRKO female mice (n=8). Values are means ±SEM as determined by a two tailed Students T-test NS, no significance.

Figure 2.11



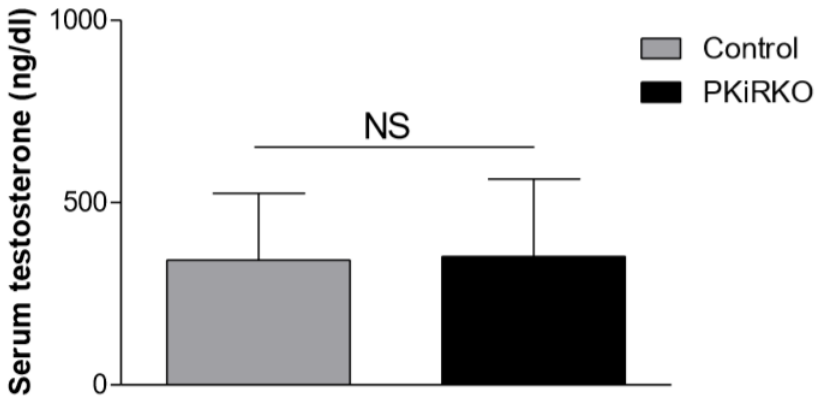
(A) Representative sections of testes. Seminiferous tubules from a Control mouse (Left side images) and a PKiRKO mouse (Right side images) show all stages of spermatogenesis with numerous sperm. (B) Representative sections of ovaries. Representative sections from a Control ovary (Left side images) and a PKiRKO ovary (Right side images) showing follicles at all stages of development, including primary, preantral, antral, preovulatory follicles as well as corpora lutea. The number of corpora lutea were also not significantly different between control and PKiRKO female mice.

Figure 2.12



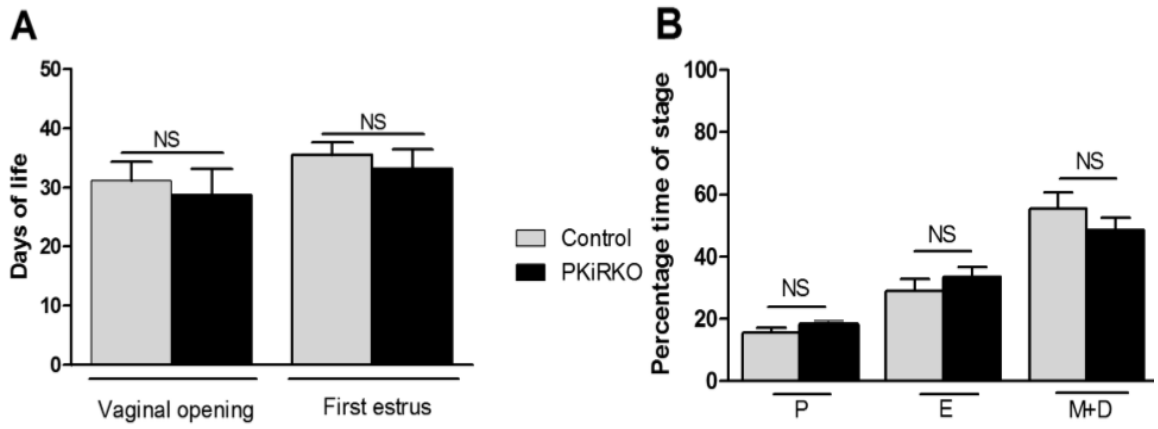
(A) Body weight change over time was not different between PKiRKO and control female (n=15). (B) The weight of ovaries were not significantly different between PKiRKO and control female mice (n=6). Values are means ±SEM as determined by a two tailed Students T-test NS, no significance.

Figure 2.13



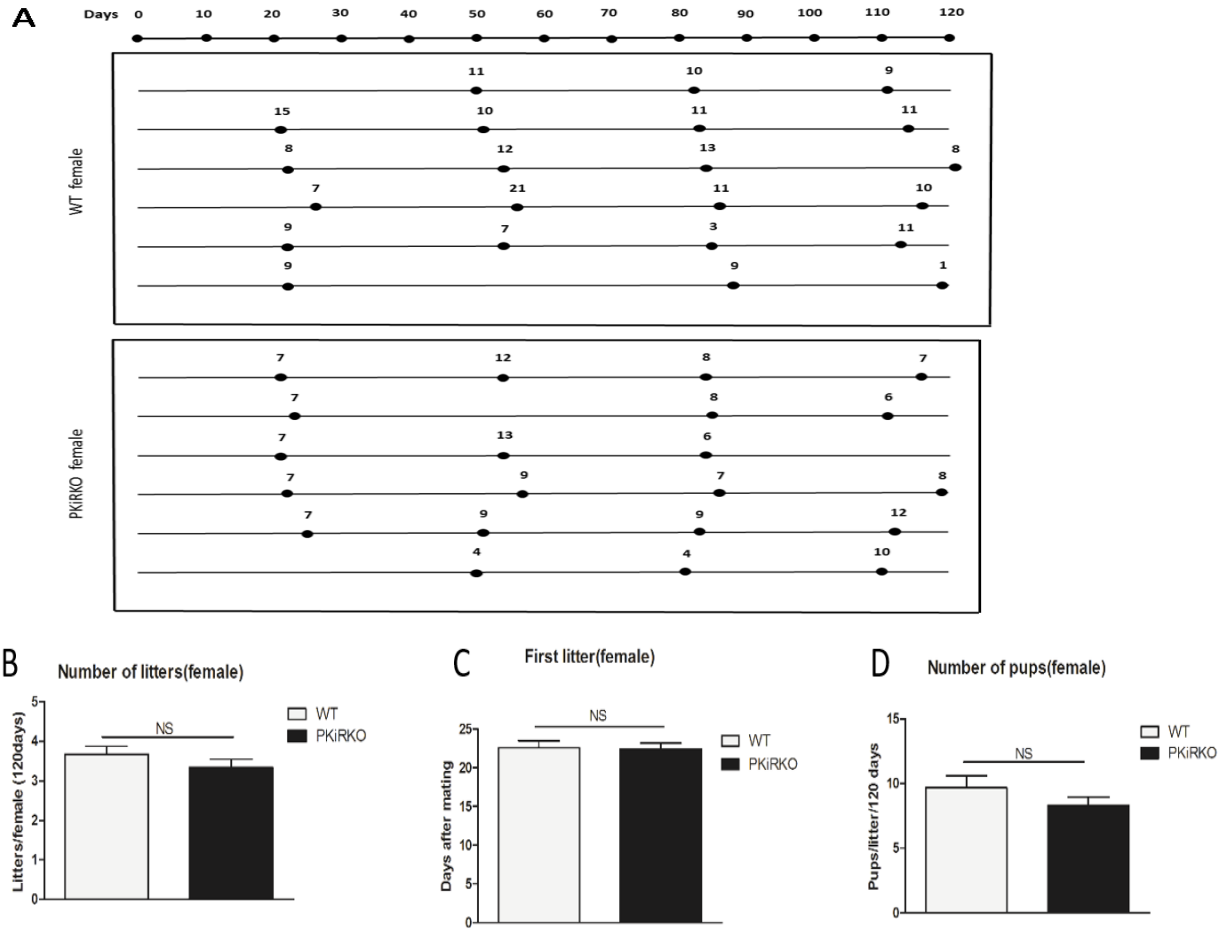
Serum Testosterone (ng/dl) measured in male control and PKiRKO mice yielded no differences. Values are means ±SEM as determined by a two tailed Students T-test NS, no significance.

Figure 2.14



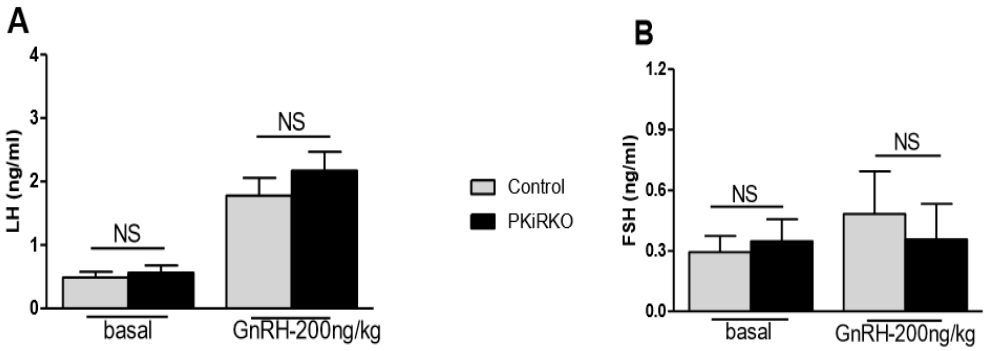
(A) VO is an estrogen dependent process that serves as an external Kisspeptin receptor in the pituitary gonadotroph of male mice indicator of female puberty onset [21]. Another measure of puberty in female mice is the age at which first estrus is observed following VO. No difference was observed at first estrus (35.6±2.4 [control] vs 33.3±3.2 [PKiRKO]) (B) Estrous cyclicity was evaluated. Vaginal smears were obtained daily over a period of 14 consecutive days in 6- to 11-week-old mice and cellular morphology examined under microscope to determine the stage of the estrous cycle. Control and PKiRKO groups both exhibited regular estrous cycles and did not differ from each other in percentage time spent at each cycle stage. Values are means ±SEM as determined by a two tailed Students T-test NS, no significance.

Figure 2.15



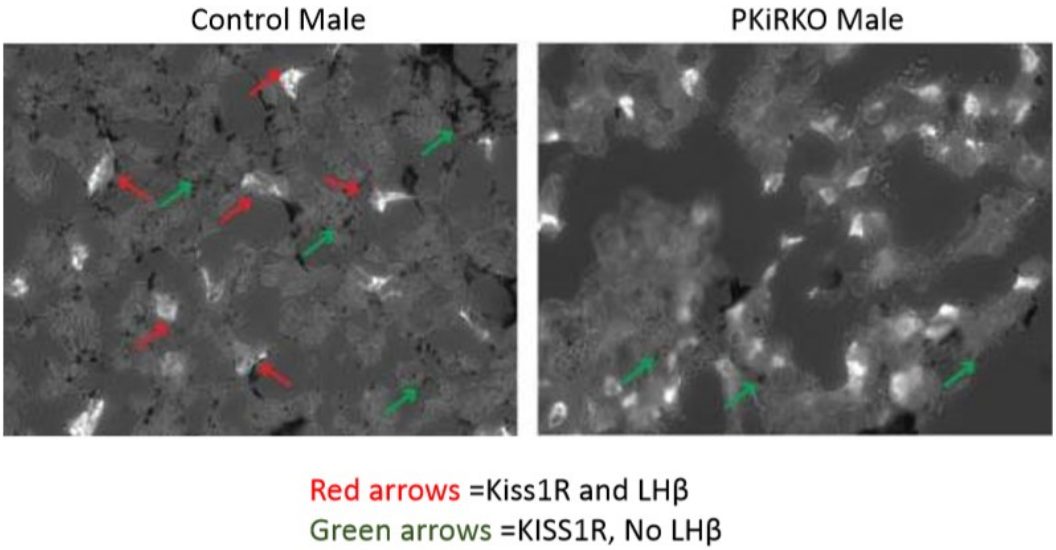
(A) To quantitate the fertility of female PKiRKO mice relative to controls, 6 control and 6 PKiRKO female mice were housed with WT male mice for 14 consecutive days (3 females and 1 male per cage, total 4 cages) and then were separated. Females were followed for an additional week until they gave birth or were assessed to not be pregnant. The same males were rotated between the 4 cages. (B) Total numbers of litters per female was not significantly different between control and PKiRKO mice during the 120 days. (C) After introduction with WT male, the day of first litter was recorded in both groups. (D) Number of pups per litter was also not significantly different between control and PKiRKO mice. Values are means ±SEM as determined by a two tailed Students T-test NS, no significance. NS, not significant.

Figure 2.16



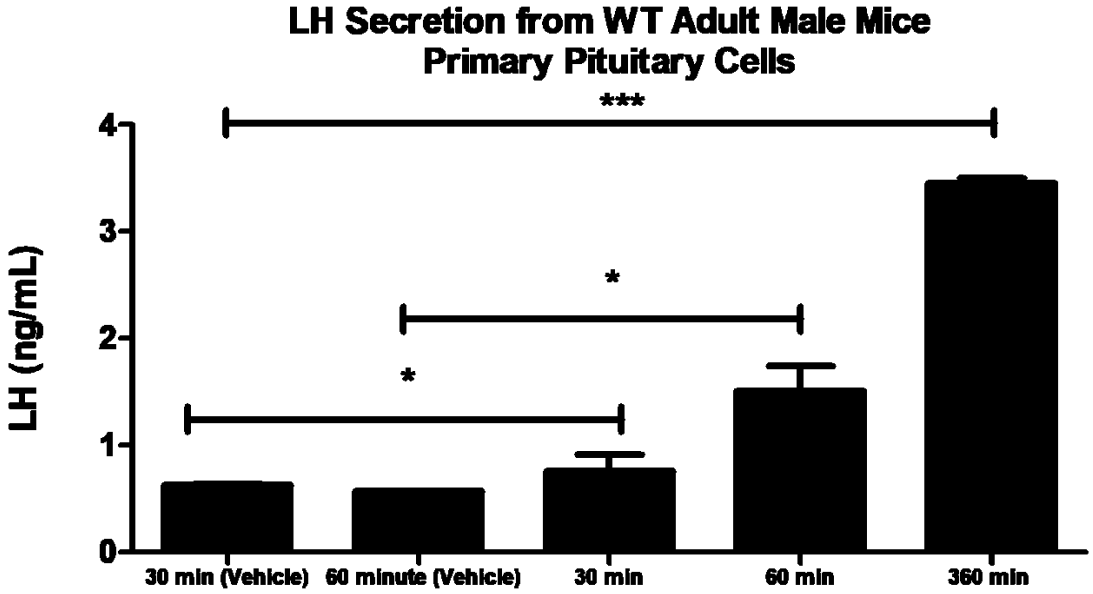
(A) Basal serum LH values in PKiRKO mice were not significantly different in females relative to control mice. PKiRKO female: 0.57± 0.27ng/mL, n=6 and Control female: 0.49±0.22ng/mL, n=6, P＞ 0.05. (B) Basal serum FSH values in PKiRKO mice were also not significantly different in females relative to Control female. Values are means ±SEM as determined by a two tailed Students T-test NS, no significance.

Figure 2.17



Double immunohistochemistry was performed for LHβ (immunofluorescence appears as white signal) and KISS1R (Nickel/DAB appears as black) Kisspeptin receptor in the pituitary gonadotroph of male mice from a representative male control mouse (left) and PKiRKO mouse (right). Red arrows point to KISS1R signal on LHβ expressing cells. Green arrows point to KISS1R signal on non- LHβ expressing cells. LHβ; Rabbit-anti Rat, NIDDK-AFP 571292393, 1:5000. KISS1R-Abcam, 1:3000. Magnification is 40x.

Figure 2.18



Ex Vivo pituitary cell culture. Pituitary cells from control male mice were treated with 30nM GnRH, for a duration of 30, 60 and 360 mins (n=4 wells per treatment). Secreted LH levels were then assessed from the cell media. Values are means ±SEM.*P≤0.05;***P≤0.001 as determined by a two tailed Students T-test NS, or one way ANOVA followed by Tukey's *post-hoc* test.

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Chapter 3

Estrogen downregulates Hepatic Kisspeptin and metabolic enzyme response

ABSTRACT

The role of Estrogen and Kisspeptin action has been limited to its powerful effects on mammalian reproduction and development. However more evidence suggests that both Estrogen Receptor (ESR1) and Kisspeptin (KISS1) signaling exert powerful effects on organ systems such as the liver and the pancreas which are outside of the reproductive axis. There is now increasing recognition that ESR1 regulation is critical for proper metabolic functions such as hepatic gluconeogenesis and lipogenesis, just to name a few. How both ESR1 and KISS1 signaling affect each other outside of the reproductive axis is not well understood. It has been previously shown that Estrogen is the main regulator of Hypothalamic *Kiss1* expression in the Anteroventral periventricular nucleus (AVPV) as well as the Arcuate Nuclei. It was thus hypothesized that estrogen could affect hepatic KISS1 function as well. After using a real time polymerase chain reaction to first determine which KISS1 transcript variant is expressed in the liver, we then performed a chromatin immunoprecipitation to confirm the presence and binding of ESR1 to the hepatic *Kiss1* gene. Furthermore, we identified functional Estrogen Response Elements (ERE's) in the liver promoter regions of *Kiss1,* which can be modulated via the addition of 17β-Estradiol (E2). Using adeno-associated virus expressing CRE recombinase to ablate hepatic ESR1 in mice, we demonstrate that there was a significant negative correlation between ESR1 expression and hepatic *Kiss1* expression. In vitro studies further showed that addition of exogenous estrogen caused KISS1 protein levels to be dramatically reduced. We further examined the effect of E2 on hepatic *Kiss1* expression as well as Glucose6 Phosphatase (*G6pase*), Phosphoenolpyruvate carboxykinase (*PCK1*), Fatty Acid Synthase (*Fas)* and Acetyl-CoA carboxylase (*Acc1*). QPCR results showed that estrogen reduced mRNA expression of *G6pase* and *PCK1,* while the lipogenic genes *Fas* and *Acc1* remained unchanged. These studies lead us to believe that hepatic kisspeptin is responsive to estrogen in a similar manner as demonstrated in the brain. With the increasing advent usage of β-Estradiol and kisspeptin as clinical therapeutic agents, their effects on metabolic parameters such as liver function must be assessed and taken into consideration.

Introduction

In recent years, the rise of public health issues related to energy imbalances such as obesity, diabetes, and metabolic syndrome have created a great burden on health care systems worldwide. In particular, the rise in the development of type 2 diabetes, metabolic syndrome and polycystic ovary syndrome (PCOS), all of which are associated with reproductive dysfunction are of great interest to researchers. In this study we attempt to further elucidate the signals that link reproductive function to metabolic status, by implicating the role alpha estrogen receptor (ESR1) has on hepatic Kisspeptin (Kiss1) expression as well as on key gluconeogenic and lipogenic genes.

Estrogen in its primary role as a female sex hormone is responsible for the regulation of both male and female reproductive development as well as secondary sex characteristics (Alonso & Rosenfield, 2002; Bakker, 2019) Estrogen exerts its effects on cells and tissues by binding to alpha-estrogen receptor (ESR1 or beta-estrogen receptor (ESR2). It is important to note that ESR1, but not ESR2, is expressed in the liver (Matic et al., 2013). Following E2 binding to ESR1, this receptor complex then directly crosses into the nucleus of cells. Once inside the nucleus this estrogen and receptor complex binds to DNA nucleotide sequences known as estrogen response elements (ERE) to regulate gene transcriptions (Ayaz et al., 2019). It has been reported that the consensus ERE of 5'GGTCAnnnTGCACC-3' when placed upstream of ESR1 responsive genes, affected the responsiveness of that gene to 17β-Estradiol (E2) (Driscoll et al., 1998) . ESR1 binding studies found in genomic searches, show that a large amount of ERE'S contains half-site EREs or ½ERE: GGTCA. These half sites were used in this study to look at their regulation of the KISS1 gene. Outside of its effects on reproduction, awry E2 and ESR1 signaling has been implicated in metabolic issues including obesity (Della Torre et al., 2016 ; Stubbins, Holcomb, Hong, & Nunez, 2012) , metabolic dysfunction (Keene et al., 2008) and increased risks of chronic diseases (Gallagher et al., 2007) (Seminara et al., 2003).

While originally discovered by the cancer field, when it was called metastin, kisspeptin (Kiss1) has been primarily studied by reproductive neuroendocrinologists due to its clear and powerful effects on the GnRH neuron. Kiss1-expressing neurons reside in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of the hypothalamus and send neuronal projections to the Gonadotropin Releasing Hormone (GnRH) neurons. KISS1, via signaling through its G-protein coupled receptor KISS1R, (Kisspeptin receptor, formerly GPR54) has been shown to be a key regulator of pubertal onset, homeostatic regulation of the HPG axis, and generation of the preovulatory LH surge (Irwig et al., 2004; Roa et al., 2006). The relationship between ESR1 and KISS1 signaling has been established in the brain. A pivotal study by Tomikawa et al showed that in the AVPV, the response of ESR1 to E2 treatment is to be recruited to the proximal promoter of the *Kiss1* gene contributing to an increase in *Kiss1* expression. While in the ARC, the ESR1 is dismissed from the proximal promoter in response to E2 leading to a decrease in *Kiss1* expression (Song et al., 2014). Characterization of hepatic KISS1 and how E2 impacts hepatic *Kiss1* expression is of focus in this study.

The role and regulation of KISS1 from non- hypothalamic (peripheral) sources is only beginning to be understood. Tolson et al. showed that adult whole body Kiss1r KO females displayed dramatically higher BW, leptin levels, adiposity as well as were glucose intolerant (Tolson et al., 2014) . The observation that triggered interest in hepatic KISS1 was based on studies by our lab to identify hepatic signals mediating impaired insulin secretion in a state of constitutive hepatic activation of PKA signaling (L-Δ-prkar1a mice). A differential gene expression screen identified KISS1 as the most highly up-regulated gene in the liver that coded for a secreted protein (Song et al., 2014) Interestingly humans with Type 2 Diabetes Mellitus also exhibit increased liver and plasma KISS1 levels (Song et al., 2014) . This finding has assisted to expand the role and responsibilities of peripheral KISS1 because it established that hepatic KISS1 is an integral component of an endocrine/metabolic regulatory loop that includes the pancreas, circulating glucose and the liver. (Song et al., 2014) Our lab recently expanded our knowledge of ESR1 by finding a role for hepatic ESR1 in glucose and lipid metabolism in male

mice including the finding that ESR1 inhibits gluconeogenesis by transcriptional inhibition of gluconeogenic genes Pck-1 and G6Pase (Song et al., 2014). Given the well-established role of KISS1 in regulating reproduction and our observations defining a role for hepatic KISS1 in regulating glucose stimulated insulin secretion, hepatic KISS1 could serve as a regulatory interface between the reproductive and metabolic systems in the body. Thus, its paramount to characterize the effect of estrogen on hepatic KISS1 as well as on liver function.

Materials and Methods

Animals

Adult male and female mice (>2 months old) were used in this study. All animal studies were carried out in accordance with National Institutes of Health guidelines on animal care regulations and were approved by the Animal Care and Use Committee of the Johns Hopkins University. Mice were maintained under constant conditions of light and temperature (14: 10 h light⁄dark cycle; 22 C) and were fed a normal chow and water ad libitum. Male flEsr1 mice, harboring LoxP sites flanking exon 3 of the Esr1 gene were injected, via the tail vein, with an adeno-associated virus expressing CRE recombinase or a control vector expressing GFP (AAV8.TBG.PI.-Cre.rBG, or AV8.TBG.PI.eGFP.WPRE-.bGH, respectively, both obtained from the Penn Vector Core). Seven days after tail injection, mice were sacrificed for further tissue processing.

HepG2 Hepatocyte Cell Culture

Hepg2 cells from a human male liver carcinoma were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 25 mM glucose, 5 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY, USA). Cells were grown and maintained in an atmosphere with 5% CO2 at 37 °C. Cells were treated with 10nM of β-estradiol (Cayman Chemicals, Ann Arbor, USA) for 12 hours.

Polymerase Chain Reaction (PCR)

DNA liver samples from male and female adult mice after feeding or fasting were subjected to PCR. In order to identify hepatic Kisspeptin transcripts, two microliters of DNA were mixed with DreamTaq Green PCR Master Mix (Thermo Scientific, Waltham, MA). as well as the Transcript Variant (TV) primers located in Supp Table 1.As previously described in (Novaira, Ng, Wolfe, & Radovick, 2009) PCR conditions are as followed; initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (59 °C, 30 s) and extension (72 °C, 45 s). The reaction was concluded with an extension for 10 min at 72 °C. The identities of amplified products were observed by agarose gel electrophoresis (1.5% agarose in buffer containing 40 mM Tris/acetate and 1 mM EDTA) and visualized with ethidium bromide staining (0.5 μg/ml) under ultraviolet light.

Chromatin Immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) was performed using ChIP-IT Express kit as indicated by the manufacturer (Active Motif, Carlsbad, CA). Liver samples from male mice which had either been kept on normal chow or fasted overnight were collected and subjected to 1%formaldehyde treatment. Using glycince, the fixation was halted. Following brief homogenization, the lysate was centrifuged at 1000 rpm for 5 min at 4°C. The nuclear pellet was then resuspended in lysis buffer and left on ice for 30 min. In order to fragment the DNA of these nuclear samples, a digital sonfier Model 250 &450 (Danbury, Connecticut were used. Samples were sonicated on ice for 4 20 sec cycles with a 60 second pause between each cycle. All samples were sonicated at 10% amplitude. The supernatant which contained the DNA fragments were them imunoprecipated using IgG ,CREB (cyclic AMP response element) ,and ESR1 antibodies as described in Supp. Table 3. Samples were then stored at -80°C. The input DNA represents an aliquot of the sheared DNA which has not been immunoprecipitated, but reverse-cross-linked/DNA cleaned up. Finally primers for mouse *Kiss1* (spanning putative ERE and CRE-containing regions) were used for PCR.

Reporter Plasmid Construct and Luciferase Assay

PCR was used on mouse liver tissues to amplify multiple fragments of the kisspeptin promoter. The primers used to amplify the different promoter regions are listed in Supp. Table 1. Added to the end of each fragment via PCR was a HIND III restriction enzyme site. The plasmid +180bp contains no ERE sites, +1102 contains 3 ERE half sites and +2956 contains 5 ERE half sites. Each fragment was then cloned into a pA3LUC reporter vector (LUC) using the common HIND III restriction site. The pCI Vector (Promega, Madison, WI) contains the CMV immediate-early enhancer/promoter region and is constitutively expressed in mammalian cells, thus it was used a positive control. An empty pA3LUC reporter was used as a negative control. All plasmids were co- transfected with the pRL Renilla (Rluc) control reporter vector and were normalized to Rluc levels. Plasmids were next co-transfected into HepG2 cells in a 96 well plate transfection protocol via Lipofectamine 2000 (Thermo Scientific, Waltham, MA). Twenty four hours after transfection, cells were treated with or without 10nM E2 for 12 hours. Following manufacture instructions, the Dual Glo luciferase assay system (Promega, Madison, WI) was used to measure relative light output (RLU) directly from the wells.

Immunofluorescence

In order to confirm ESR1 knockdown via tail vein injection, liver tissue were harvested from injected mice. The liver was then fixed in 4% paraformaldehyde overnight. The following day the liver tissue is immersed in 30% sucrose for 24 hours at 4 °C. Finally, the liver tissue is embedded in Tissue-Tek O.C.T (optimum cutting temperature) compound. Samples were next sent to the Johns Hopkins Reference Histology Reference Core for sectioning (5 um). As previously described, (Qiu et al., 2017a) immunofluorescence required the liver tissue to be treated with 1% Triton and blocked in 5% goat serum. Next the samples were immersed in 1% goat serum containing a 1:4000 dilution of anti-ESR1 rabbit (Millipore, California, CA). Slides which contain the liver tissue were incubated with Alexa Fluor 594 goat anti-rabbit IgG (H + L) for 1 hour before nuclear counterstaining with DAPI and mounting (Vectashield Vector laboratories, Burlingame, CA). ESR1 fluorescence intensity as a measure of "protein density" was analyzed using ImageJ (National Institute of Health).

RNA Isolation and cDNA Synthesis

Total RNA was extracted from a 6 well plate of HepG2 cells using the TRizol Reagent (Invitrogen, Carlsbad ,California, USA) following the manufacturer's instructions. Briefly, after a short incubation with the TRizol reagent, the cells were lysed using an homogenizer. The aqueous phase was separated by centrifugation after adding chloroform. The RNA was next precipitated as a pellet using isopropyl alcohol. The pellet is then washed with 75% ethanol, spun down again and finally resolubilized in nuclease-free water. RNA samples were quantified by using a Epoch spectrophotometer (BioTek Instruments, Winooski, VT). 1micorgram of RNA was converted to cDNA synthesized using the iScript cDNA Synthesis Kit (Bio-Rad).

Quantitative Real-Time PCR (q-RT-PCR)

Relative cDNA levels were determined using SYBR Green master mix Solution (Bio-Rad,Hercules, CA, USA). The genes of interest as labeled in Supp. Table 2.1 were examined by q-RT-PCR using an I-Cycler quantitative PCR machine (Bio-Rad). For each gene, 18S rRNA was used as a control. Reactions were carried out according to the manufacturer's instructions and conditions. Briefly, the first denaturing step is at 95 °C for 3 min, the second denaturing step was then 35 cycles at 95 °C for 10s. The annealing step required primer temperature ranges of 55–61 °C for 20s (depending on primer set). The final extension step was for 72 °C for 30 s. For each primer set, PCR efficiency was determined by measuring a 10-fold serial dilutions of cDNA and reactions with between 95% and 105% PCR efficiency were included in subsequent analyses. Relative differences in cDNA concentration between baseline and experimental conditions were then calculated using the comparative threshold cycle (Ct) method. Briefly, for each sample, a ΔCt was calculated to normalize for the internal control using the equation: $\Delta Ct = Ct(gene) - Ct(18S)$. To obtain differences between experimental and control conditions, ΔΔCt was calculated: $\Delta Ct(sample) - \Delta Ct(control)$. Relative mRNA levels were then calculated using the equation fold difference = $2^{-\Delta\Delta Ct}$.

Western Blot

HepG2 cells were cultured in a 6 well plate and grown to confluency. Cells were then treated with estradiol for 12 hours. Cells were then lysed and harvested in RIPA buffer (Cell Signaling

Technology, Danvers, MA) buffer supplemented with protease and phosphatase inhibitor (Thermo Scientific, Waltham, MA). Equal amounts of proteins(30μg) from the cell lysates were then separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (Thermo Scientific, Waltham, MA) and transferred to nitrocellulose membranes via a Trans Blot Turbo machine (Bio-Rad, Hercules, CA, USA).Next, the membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) at room temperature, followed by an overnight incubation with the primary antibody anti-kisspeptin (Millipore, California, CA) at a 1:1000 dilution as shown in (Table 3). The blot was then incubated in goat anti-rabbit (IgG-HRP conjugate) secondary antibody (Bio-Rad, Hercules, CA, USA) at room temperature for 2 hours. The blots were then washed and protein bands were detected using enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA). Densitometry was quantified using Image J (National Institutes of Health)

Data and Statistical Analysis

Each experiment in each experimental group was performed using either triplicate samples (Luc Assay or duplicate samples (q-RT-PCR)We used an unpaired Student's *t*-test to compare the mRNA levels of genes between groups treated with estradiol or vehicle. All results are expressed as mean ± SEM and $p \leq 0.05$ assigned as significant using the GraphPad Prism 4 software (San Diego, CA, USA).

Results

Hepatic Kiss1 Gene and its transcript variant.

The *Kiss1* gene (Fig1A) consists of a number of splice variants that produce the same protein product(RW.ERROR - Unable to find reference:1460; RW.ERROR - Unable to find reference:1792) (Castellano, Wright, Ojeda, & Lomniczi, 2014) . However these published splice variants are from hypothalamic sources. In order to determine which transcript variant was found in the liver, we performed PCR using multiple transcript variant (TV) primers labeled in Table 1. Using male and female livers that had been fed or fasted as our DNA template, PCR was performed and ran on a polyacrylamide agarose gel. As shown in the gel the only transcripts that are visible are Transcript

Variant (TV) 4 and TV 4* around 600 base pairs. This leads us to believe that hepatic KISS1 expresses the full length KISS1 product identified as Ensembl Kiss 001 and 002.

CREB AND ESR1 bind to hepatic Kiss1 Promoter

Chromatin immuno-precipitation (ChIP) analysis of the upstream regions of Hepatic *Kiss1* was used to explore ESR1 interaction with an E2 response element (ERE) in the proximal *Kiss1* promoter. We used ChIP to explore ESR1 occupancy on the ERE in the *Kiss1* promoter (Figure 3.2) in the liver of mice fasted overnight, or control mice fed ad libitum. As previously reported (Castellano et al., 2014), glucagon induces CREB occupancy on a CREB response element (CRE) in the *Kiss1* promoter in response to a fast. Supporting the observation that Esr1 expression was lower in fasted and DIO mice, we observed a dismissal of ESR1 from the *Kiss1* promoter in response to fasting suggesting that ESR1 serves to repress Kiss1 expression in the liver.

Estrogen affects Kiss1 promoter activity

To explore the protein-DNA interactions that occur within the Kiss1 promoter and the role of these interactions in targeting hepatic Kiss1 gene expression we created fragments of hepatic kisspeptin promoter (+180, +1102, and +2956) fused to the luciferase reporter gene. Up to 5 ERE's on the Kisspeptin promoter are found on the +2956 plasmid and 3 ERE's on the 1102 promoter, while the shortest fragment +180 promoter contains no ERE's and thus was utilized as a negative control. After transfections of plasmids and treatment of HepG2 cells with 10nM of β-Estradiol treatment for 12 hours, results show E2 caused a significant increase in relative light output only in the +2956 Kisspeptin promoter plasmid (Fig 3). There was no significant change in the +180 and +1102 group. The positive control and negative controls behaved as expected as a high RLU output was detected in the +/- E2 pCI group, and a low output in the empty LUC group, respectively.

Negative correlation between ESR1 expression and hepatic Kiss1 expression

In order to determine the effect of ESR1 on hepatic Kiss1 expression, we deleted ESR1 in the livers of live mice. Male fl*Esr1* mice were created by Chen et al (Chen et al., 2009) and harbor LoxP

sites flanking exon 3 of the *Esr1* gene. These mice were tail injected, with either an adeno-associated virus expressing CRE recombinase (called LERKO mice going forward) or a control vector expressing GFP. In order to quantify the knockdown of hepatic ESR1 expression mice were sacrificed and liver harvested for histological analysis. LERKO mice exhibited nearly complete disruption of *Esr1* mRNA levels and ESR1 protein levels compared to GFP injected controls as shown in Figure 3.4 B and quantified in Figure 3.4 A and C. LERKO mice were found to have a dramatic increase in *Kiss1* mRNA levels which was highly inversely correlated with *Esr1* mRNA levels (Fig 5a and b), and a nearly 30% increase in serum KISS1 (Fig 3. 5c)

E2 reduces kiss1 protein and mRNA levels in vitro

To further validate the negative correlation between ESR1 and Kiss1 expression we directly treated HepG2 cells with exogenous E2 and looked at effects on both protein (Fig 3.6 A & B) and mRNA levels (Fig 3.6 C). We show that both 1nM and 10nM were effective in significantly reducing KISS1 expression in hepatocytes. Normalizing with actin protein levels, KISS1 expression levels are quantified in Figure 3.6 B. Furthermore, qPCR results show that *Kiss1* mRNA levels were significantly reduced when HepG2 cells were treated with 10nM of E2 for (Fig 3.6 C).

E2 reduces hepatic gluconeogenic genes and leaves lipogenic genes unchanged

In a novel study using primary hepatocytes from LERKO mice, Qui et al (Qiu et al., 2017) showed that E2 treatment lead to the downregulation of the major gluconeogenic genes *PCK1* and *G6Pase* as well as de novo lipogenic genes *FAS and ACC1.* In order to validate these effects in an in vitro cell model (HepG2), we also measured mRNA expression changes in the aforementioned genes. Figure 3.7 A and B show that HepG2 gluconeogenic genes were both significantly reduced by 12 hours of E2 treatment. Surprisingly we did not see decreases in de novo lipogenesis in HepG2 cells. Rather we saw no significant changes in *FAS* (Fig 3.7 C) or *ACC1* (Fig 3.7 D). Possible reasons for this difference between primary hepatocytes and cell models will be expanded upon later.

Discussion

Estrogens have been shown concussively to control the development and physiology of mammals. While the role and study of estrogen signaling has been limited to the development and function of the female reproductive system, there is mounting evidence that they also play crucial roles in male reproduction and in tissues outside of the reproductive axis such as the liver (Qiu et al., 2017b), adipose tissue, cardiovascular system and adipose tissue (Couse & Korach, 1999; Ciocca & Roig, 1995). Of focus in this study is the effect of E2 on the newly discovered hepatic hormone KISS1. We have recently reported that the liver is a source of circulating KISS1 and that hepatic KISS1 serves to regulate glucose stimulated insulin secretion (Song et al., 2014) This profound finding leads to a plethora of questions addressing the regulation of hepatic KISS1. Hepatic KISS1 and its role beyond a paracrine hormone from the liver are just beginning to be understood.

Although several studies have looked at the regulation of *Kiss1* expression (Tomikawa et al., 2012; Castellano et al., 2014; Mueller et al., 2011) , these studies have utilized brain and reproductive tissues to profile *Kiss1,* thus leaving knowledge of the transcript variant expressed in the liver unknown. Using liver samples from both male and female fed and fasted mice we conducted PCR to identify which KISS1 transcript is expressed in the liver. As noted in Table 1, we utilized publicly available NCBI and Ensembl *Kiss1* transcript primers to identify our liver *Kiss1.* Our results as shown in Figure 3.1 B are that hepatic KISS1 expresses the full length KISS1 product identified as Ensembl Kiss 001 (TV 4) and 002(TV4*). Interestingly other predicted transcript variants such as the shorter 202 (TV 1) and the longer 201 (C.V.) were unable to be detected via PCR. It was noted that male PCR products in Figure 3.1 B showed several bands in there lanes, which were not seen in the PCR product bands of female hepatic liver samples. This difference in *Kiss1* PCR products between male and female livers could be indicative of a sexual dichotomy in *Kiss1* hepatic splice variants. However, taken together we believe the prevalent band seen around ~650 bp is the hepatic *Kiss1* transcript *Kiss1 001* and 002.

The analysis of *Kiss1* expression in mouse is complicated by reports of at least five different transcript variants expressed under the control of different promoters (Figure 3.1 A). While all the transcripts produce identical KISS1 protein, differences in promoter regulatory elements could contribute to tissue specific differences in regulated *Kiss1* expression. For example, CREB regulation of *Kiss1* in the brain is mediated by a CRE in the promoter of transcript variants 001 and 002 (Song et al., 2014) and ESR1 regulates hypothalamic expression of transcript variants 201 and 202 (Tomikawa et al., 2012). A similar finding was reported for expression of *Kiss1* in the rat (Castellano et al., 2014). We observed a reduction of ESR1 on the *Kiss1* promoter in response to fasting. This effect was reversed after feeding. This evidence begins to suggest that ESR1 may repress *Kiss1* expression in the liver alluding to the fact that hepatic *Kiss1* is regulated by both metabolic status and reproductive status, and may in turn regulate metabolic and reproductive function.

To further understand the transcriptional regulation of hepatic *Kiss1,* in vivo epigenetic analyses of hepatic *Kiss1* promoter region was conducted. Work from our group (Song et al., 2014) has shown that one of the known promoter regions of *Kiss1* contains a cyclic AMP response element (CRE), suggested to mediate glucagon regulation of Kiss1, while another promoter contains an estrogen response element (ERE). The CRE and ERE in the Kiss1 promoters are found in the proximal regions before Exon 2 and 3 respectively (Figure 3.1 A). Promoter reporter vectors were developed by inserting promoter fragments into the PA3LUC reporter vector (+180, +1102, and +2956). All reporter fragments, but the +180bp promoter fragment include an ERE. Transfections were performed in the presence or absence of E2 to identify E2 responsive regions specific to hepatocytes (HepG2) as assessed by luciferase activity. As shown in Figure 3.3, after normalization to Rluc levels, the +2956 KissProm vector yielded the most significant increase in RLU values after addition of 10nM of E2. This suggests that during the fed state (DMEM containing serum), the addition of exogenous E2 was able to modulate the expression of hepatic *Kiss1*.

The production of the LERKO mice gave us clear evidence that E2 was indeed regulating hepatic *Kiss1* expression. After ablating hepatic ESR1 via a viral tail vain injection targeting liver ESR1, we showed that both *Kiss1* mRNA and serum KISS1 levels were significantly increased (Figure 3.5. This increase in serum KISS1 was associated with a ~2-fold attenuation of glucose stimulated insulin secretion (Ratio of glucose stimulated insulin secretion to basal, AAV8-GFP, 12.27 +/- 2.9, AAV8-CRE, 6.24 +/-1/46, $p<0.05$). These data support a negative regulation of *Kiss1* expression by ESR1.Contrary to both the ARC and AVPV, where ESR1 interaction with the *Kiss1* promoter up-regulates *Kiss1* expression; E2 increasing promoter occupancy by ESR1 in the AVPV and decreasing promoter occupancy by ESR1 in the ARC (Tomikawa et al., 2012; Goto et al., 2015). Because the presence of ESR1 serves to repress *Kiss1* expression we believed that direct treatment of the liver with E2 should also directly cause *Kiss1* expression to decrease. Figures 3. 5 A, B and C show the effect of E2 on hepatic KISS1 and *Kiss1* mRNA levels. The dramatic decrease of hepatic KISS1 post E2 treatment further implicates further how reproductive status could affect metabolism.

The liver plays a crucial role in glucose and insulin homeostasis. Not only does the liver itself produce hepatic glucose, but it also sends cues to other organs i.e KISS1 to the pancreas, in order to modulate glucose production. It has been shown that estrogen deficiency contributes to the development of hyperglycemia and type 2 diabetes (Gallagher et al., 2007). Previous studies in which *ESR1* has been deleted show that in both male and female ESR1 knockout mice are obese with both insulin resistance and impaired glucose tolerance present (Heine, Taylor, Iwamoto, Lubahn, & Cooke, 2000). Our data contributes to those findings as we found that the key gluconeogenic enzymes G6Pase and PEPCK are reduced in the presence of E2 (Figure 3.7 A and B). The gluconeogenic enzymes G6Pase and PEPCK do not undergo posttranslational modifications and are regulated by transcriptional activity (Jitrapakdee, 2012) . We show that E2 directly downregulates mRNA expression of *G6Pase* and *PEPCK* possibly by directly binding to the ERE's found on their promoters as suggested by Qui et al (Qiu et al., 2017) (*Pck*-2 EREs in proximal promoter & *G6pase*-2 EREs in proximal 400bp of

promoter). More epigenetic analyses such as histone and chromatin changes in the proximal promoter are needed to properly assess ESR1 regulation of hepatic *Kiss1*, *Pck1*, and *G6p1* gene expression. We saw no significant changes in the de no novo lipogenic genes *FAS* (Figure 3.7 C) or *ACC1* (Figure 3.7 D). It was previously suggested that E2 treatment of primary hepatocytes caused a decrease in the aforementioned genes (Qiu et al., 2017) . Although we see a slight decrease in *ACC1* mRNA levels, the decrease was not significant. This difference could be due to the models in which are being used, a primary hepatocytes versus a hepatic cell line. The HepG2 cell line being the most commonly used liver cancer cell line in metabolic studies was chosen for this study. However other hepatic cell lines such as Hep3B, Huh7 and HA22VT could possibly be treated with E2 and yield results closer to the published primary hepatocyte lipogenesis data.

Given the well-established role of E2/KISS1 in regulating reproduction, our previous observations defining a role for hepatic KISS1 in regulating insulin secretion, and now our finding of E2 directly affecting KISS1 expression; this evidence leads us to believe that hepatic KISS1 could serve as a mediator of signals and cues between the reproductive and metabolic systems in the body. Kisspeptin was only discovered in 2003; therefore, there are still numerous advancements and contributions to be made in the study of its action and regulation. An understanding of the tissue specific regulation of the hepatic KISS1 could contribute to the development of novel therapeutics that would target hepatic KISS1 effects regulating metabolism, while sparing precious and critical neuroendocrine KISS1 function. Additionally, comprehending ways in which reproductive status can impact energy metabolism could broaden society's understanding of metabolic changes that occur during puberty or in states of reduced estrogen levels such as menopause.

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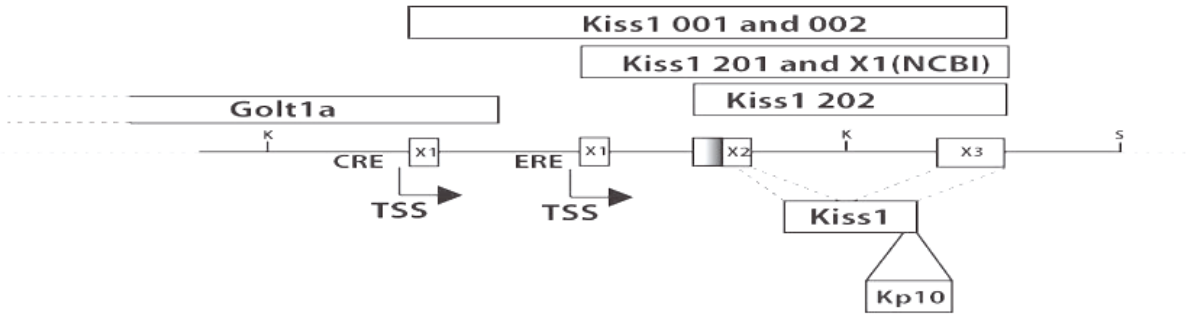
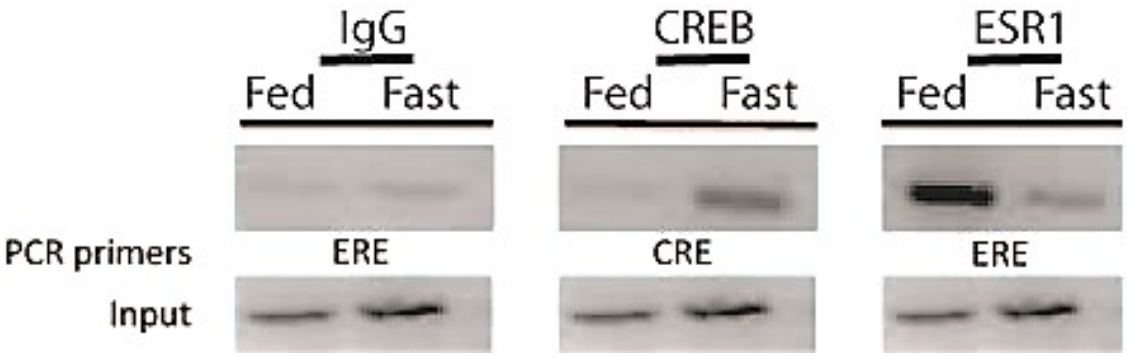
Figure Legends

Figure 3.1



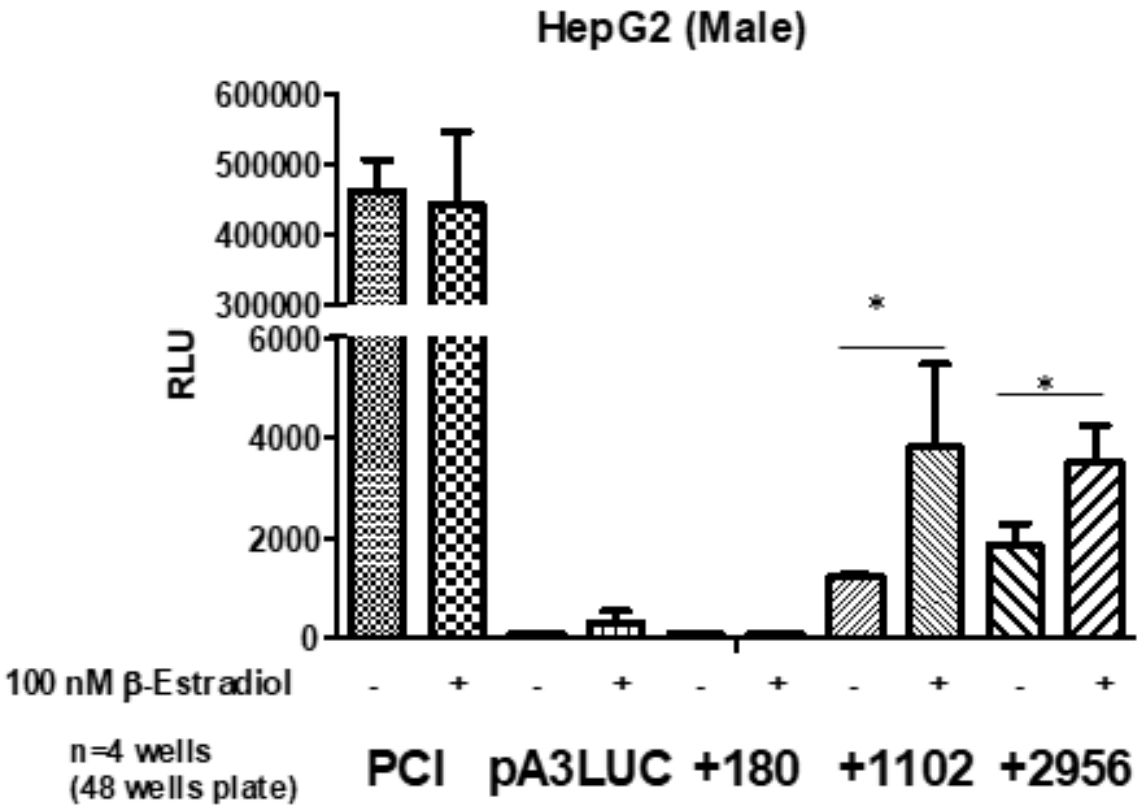
A.) Transcript variants of mouse Kiss1 are expressed from two different first exons, 001 (referred to as transcript X3, XM_006529681, on NCBI) and 002 (referred to as X4, XM_006529682, on NCBI) are regulated by CREB at a CRE and 201 (referred to as X2, XM_006529680, on NCBI) and X1 (XM_006529679, not listed at Ensembl) are regulated by ESR1 at an ERE. X1 and X3 include a larger 2ndexon (including the shaded region) and X2 and X4 include a smaller 2nd exon. The transcriptional start site for Kiss1X3and X4 is located in an exon of the Golt1a gene. **B.)** PCR was performed on fed/fasted male and female liver samples using primer pairs as labeled in Supp. Table 1. Transcript Variant (TV) 4 and TV 4* are the only Kisspeptin transcripts found in the liver.

Figure 3.2



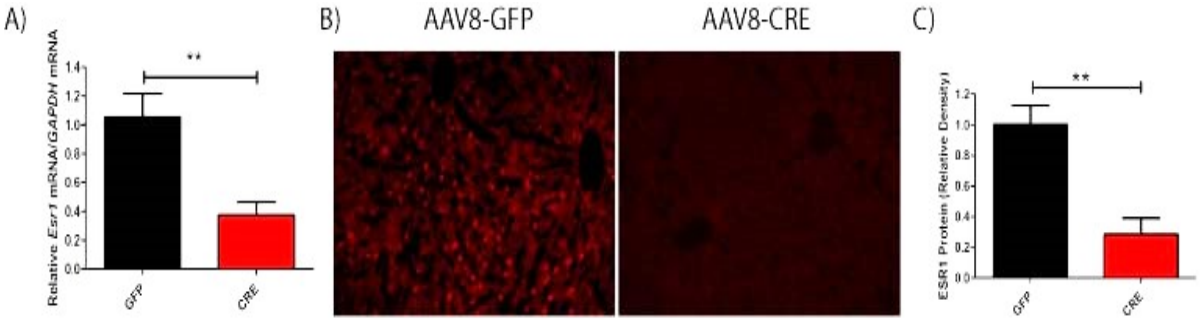
ChIP results showing increased occupancy by CREB at the CRE, and reduced ESR1 occupancy at the ERE in the Kiss1 promoter following an overnight fast in male mice. IgG used as a negative control. PCR of input DNA shown in the lower panels

Figure 3.3



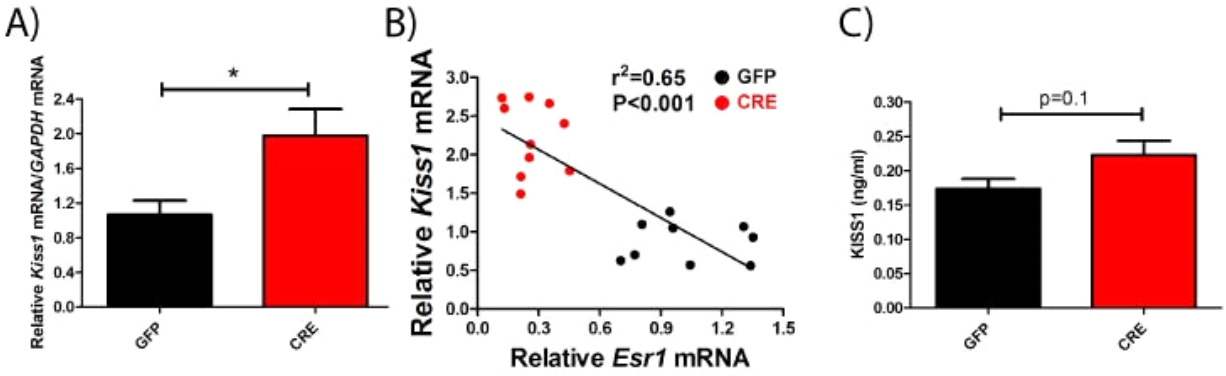
Luciferase (LUC) expression in Kiss1Prom-LUC reporter plasmids. The pattern of LUC/Rluc expression measured as relative light units (RLU) is a reflection of Kiss1 promoter activity in the presence of 10nM of exogenous E2. pCI=positive control,EmptyPa3LUC (no promoter insert),+180bp(no ERE's), +1102bp(3 ERE's), +2956bp(5 ERE's). Graphed as means ± SEM of RLU. (Three independent experiments were conducted using triplicate samples-Two Tailed-Student Test analysis was performed.)

Figure 34



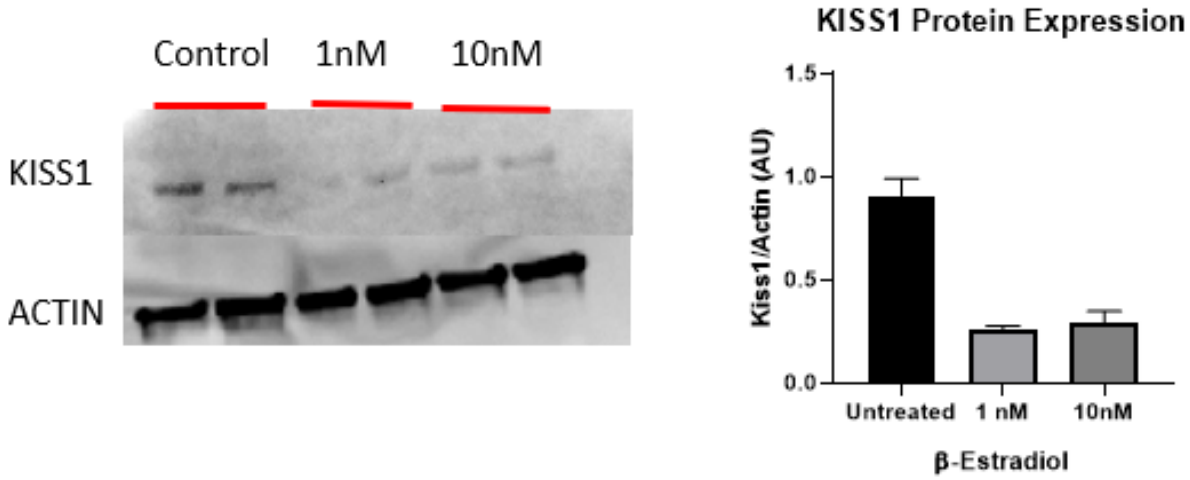
A) Relative mRNA levels of Esr1 in livers from control (black bar) and CRE (red bar) injected male mice. **B)** Liver immunohistochemistry for ESR1 protein (Red-Cy5 staining). Left is liver section from GFP injected control, to the right is liver section from CRE injected mouse. **C)** Image J analysis calculated relative levels of ESR1 Ab labeled with Cy5 in GFP (Black bar) and CRE (red bar) injected mice. For A and C, n=4-6 mice, ** indicates $p<0.01$. (Three independent experiments were conducted using triplicate samples-Two Tailed-Student Test analysis was performed.)

Figure 3.5



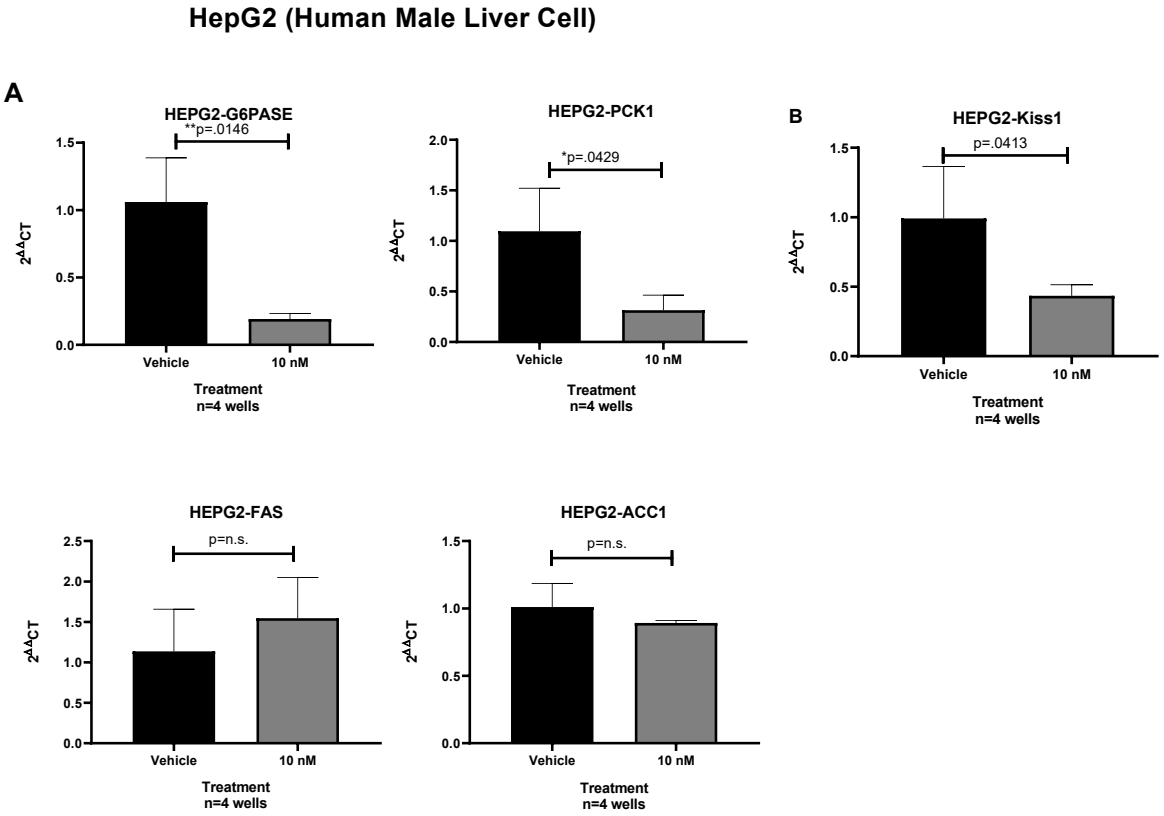
A) GFP injected control mice=black. CRE injected LERKO mice=red. Liver Kiss1 mRNA, n=4-6 mice, * indicates $p<0.05$. **B)** Correlation of Esr1 and Kiss1 mRNA in the. Pearson correlation analysis. **C)** Serum KISS1 levels as measured by RIA**.** (Three independent experiments were conducted using triplicate samples-Two Tailed-Student Test analysis was performed.)

Figure 3.6



A.) Protein expression of HepG2 KISS1 and Actin after treatment with 1 and 10nM of E2. **B.)** Quantification of the KISS1 protein band densities. (n=2 bands per treatment). **C.)** QPCR results of *Kiss1* mRNA after E2 treatment of HepG2 cells for 12 hours.

Figure 3.7



A.) Effect of E2 treatment on the mRNA levels of the gluconeogenic genes *G6pase* and *Pck1* as well as the lipogenic genes *Fas* and *ACC1* are shown. **B.)** mRNA levels of *Kiss1* in response to estradiol treatment. n=4 wells. The data are expressed as the means ± SD, *p<0.05, vehicle vs 10nM estradiol. (Three independent experiments were conducted using triplicate samples-Two Tailed-Student Test analysis was performed.)

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Chapter 4

Low-Dose DHT's effects on glucose and energy metabolism are ameliorated by central nervous system knockout of the androgen receptor in female mice.

Abstract

Hyperandrogenima (HA) and Polycystic Ovarian Syndrome (PCOS) are a result of the imbalance of androgen secretion. Due to the international scope of its effects on reproductive age women, mechanisms of HA signaling have become a key area of study. The classical defects caused by both HA and PCOS include but are not limited to the following; hyperandrogenemia, oligo/anovulation, polycystic ovaries, diabetes mellitus, hypersecretion of luteinizing hormone (LH), and hyperlipidemia. Using a low dose treatment of dihydrotestorsteorene (DHT) we are able to recapitulate impaired glucose homeostasis and hyperinsulinemia in female mice. Findings from clinical observation, animal models and pharmacological studies have provided strong evidence to support the direct involvement of androgens and androgen receptors (AR) in DHT induced metabolic and reproductive dysfunction .We and other labs have hypothesized that the androgen receptor (AR) might be a possible target for unraveling HA induced metabolic defects. In this study, we specifically ablated the AR in the central nervous system (CNS), creating a mice referred to as SynARKO (Synapsin Androgen Receptor Knockout). Characterization of the SynARKO mice included reproductive tests, hormonal assays, and metabolic tests. SynARKO mice were assessed by the ages of vaginal opening and first estrus, where no differences were seen in comparison to WT mice. There was also no difference in cyclicity and fertility between Control and SynARKO mice. When challenged with a bolus of glucose via a glucose tolerance test (GTT), female mice of Control and SynARKO implanted with DHT (3 months of DHT insertion) that are younger than five months old displayed impaired blood glucose tolerance as Con-DHT when compared to Con-no DHT mice. However, once these mice older than seven months with DHT (5 months of DHT insertion), SynARKO-DHT mice showed improved GTT compared to those of Con-DHT without improvements in the insulin resistance. This improved GTT was also followed

by overall improved metabolic rates including improved rates of oxygen intake and carbon dioxide release. Furthermore, differences in energy expenditure were observed between Control-DHT and SynARKO-DHT mice. The improvements in both GTT and metabolic rates by deleting AR in the CNS implicate that the CNS-AR has a crucial role in metabolic homeostasis of middle-aged female mice with HA.

Introduction

Hyperandrogenism is one of the diagnostic criteria of PCOS, along with anovulation and morphologic polycystic ovaries. According to the Rotterdam criteria, a patient with two of the three criteria can be diagnosed with the syndrome (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). In order to model PCOS effects in animals, several animal models have been produced in literature. These models include high dose DHT (Caldwell, A. S. et al., 2014; van Houten et al., 2012), letrozole treatment (Kauffman et al., 2015; Kelley, Skarra, Rivera, & Thackray, 2016), Dehydroepiandrosterone (DHEA) (Elia et al., 2006; Solano, Sander, Ho, Motta, & Arck, 2011) as well as prenatal androgen exposure (Demissie et al., 2008; Roland, Nunemaker, Keller, & Moenter, 2010). However, these models showed reproductive dysfunction but were also associated with obesity. This is problematic because obese female rodents and women have higher circulating testosterone levels and impaired fertility and metabolic dysfunction (Brothers et al., 2010; De Leo et al., 1998; Delemarre-van de Waal et al., 2002). Consequently, it is not possible to determine whether the phenotype is caused by the androgen excess or obesity. Further, serum androgen levels in women with PCOS or corrected congenital adrenal hyperplasia (CAH) are approximately 2-3 fold higher than in normal women (van Houten et al., 2012; Caldwell et al., 2017; Kamel & Krey 1982; Silva et al., 2018). Because of this, and in contrast o other high dose androgen

models, we created an adult mouse model with impaired reproductive and metabolic dysfunction by chronic DHT exposure from insertion of a pellet with 4mm length of crystal DHT powder (total length of pellet is 8mm). This model produces serum DHT levels that are about two fold higher (referred to as 2xDHT) than that of control mice without DHT treatment (Wu et al., 2014). These mice displayed normal body mass/composition and pathophysiological serum androgen levels (twofold that of controls). With this new low dose DHT model, Andrisse et al (Andrisse et al., 2017) demonstrated that hepatic AR with HA contributes hepatic insulin resistance and increased hepatic gluconeogenesis.

The androgen receptor is a member of the nuclear receptor subfamily and mediates the action of testosterone and dihydrotestosterone. Because of their steroidal nature, androgens can directly bind the genes and become transcription factors (Mooradian, Morley, & Korenman, 1987). AR contains both an N & C-terminal regulatory domain (Jenster, van der Korput, Trapman, & Brinkmann, 1995), a DNA binding domain (DBD), a hinge region, and a ligand binding domain (LBD) (Saporita et al., 2003). AR signaling has been shown to alter an array of genes including but not limited to; *FoxO1* (Andrisse et al., 2017a)*,CREB* (Aarnisalo, Palvimo, & Janne, 1998; Kim, Jia, Stallcup, & Coetzee, 2005)*, BRCA1*(Yeh et al., 2000)*, LH/FSHβ* (Dalkin et al., 1992; Thackray, McGillivray, & Mellon, 2006)*,SIRT1* (Fu et al., 2006)*,p300* (Fu et al., 2000) and *SRY* (Yuan, Lu, Li, & Balk, 2001) just to name a few. The role of androgens in male fertility is known and well-studied (Abbott, Padmanabhan, & Dumesic, 2006), but the role in female fertility is under investigation. A global knockout in females was reported by Hu and colleagues in 2004 and revealed that AR knockout mice have fewer corpa lutea, subfertility, and longer estrous cycles (Hu et al., 2004). Subsequent studies by Shiina et al found that AR deficient mice experienced premature ovary failure and age

dependent follicle loss (Shiina et al., 2006). In addition, a granulosa cell specific deletion of AR yielded the same phenotype of reproductive failure that is similar to that the effect of an AR global knockout (Sen & Hammes, 2010). The transplantation of WT ovaries into global KO mice did not completely restore fertility and suggested that AR causes a defect in the system that cannot be rescued with only a transplant. These results taken together indicate that AR is regulating fertility at another level, most likely the pituitary or hypothalamus.

To begin to assess the role of AR in the neuroendocrine system with or without HA, Wu et al. created a pituitary specific androgen receptor knockout to explore the role of AR in the pituitary (Wu et al., 2014; Wang et al., 2019). Deletion of AR in gonadotropes ameliorated DHT induced infertility. These results taken together indicate that AR is regulating fertility may act in multiples levels.

HA in the brain has been understudied with few studies implicating AR action in the brain. One particular study found that the neuropeptide Kisspeptin is mediated by AR and may be important in fertility. Rats implanted with DHT for 90 days had significantly less Kiss1 expressing cells in their Arcuate nuclei neuronal population, the authors hypothesized that the inhibition of Kisspeptin expression is responsible for the decreased LH and FSH surge (Iwata, Kunimura, Matsumoto, & Ozawa, 2017). Caldwell et al. used brain AR knockout female mice as well as a DHT-induced PCOS model to determine how androgens act within the brain during the development of PCOS. The conclusion was that brain-specific AR signaling with HA led to key PCOS phenotypes including dysfunctional ovulation and polycystic ovaries, as well as some metabolic problems (Caldwell, A. S. L. et al., 2017). To date, only one other group has attempted to delete AR in the central nervous system. Using male mice, Chang et al, found that neuronal AR regulates hypothalamic insulin signaling by repressing NF-Kb. This then lead to

insulin resistance, lipid accumulation, and visceral adiposity (Yu et al., 2013). This describes effects of AR function in male mice, AR function in the CNS of female mice with HA is yet to be examined.

The regulation of energy metabolism and reproduction is complex, involving multiple tissues, hormones and energy containing substrates. In this study we aim to directly investigate the role of central nervous AR in the hyperandrogenemia-induced reproductive and metabolic dysfunction.

Materials and Methods

Generation and genotyping of SynARKO mice

The central nervous system (CNS) specific knockout was created using Cre recombinase under the control of the Synapsin promoter, referred to as SynARKO. The knockout is generated by mating a floxed AR mouse (acquired through EMMA, The European Mouse Mutant Archive, deposited by Guido Verhoeven) with a Synapsin Cre mouse (Jackson Laboratories). To obtain heterozygous females, a Synapsin Cre female (SynCre+/-; AR wt/wt) was mated with a floxed AR male (AR fl/y; Cre-). To create the SynARKO mouse (AR fl/fl; cre+/-), a heterozygous female (fl/wt; SynCre+/-) was mated with a male (AR fl/y; cre-). Littermates were used as controls if they were female and had the appropriate genotypes (AR fl/wt; cre-) or (AR fl/fl; cre-). Genomic DNA isolation and primers (M28/M29) were used to detect the AR gene. Cre (M28) primer Forward: GTTTACGCTACCCCGTGCTC and (M29) Reverse: CATCTTCAGGTTCTGCGGGAAACC. All procedures were performed with the approval of the Johns Hopkins Animal Care and Use Committee.

Western Blot

Knockout and control mice were sacrificed, followed by tissue extraction of hypothalamus, pituitary, ovary, and liver. All samples were immediately frozen in liquid N2. Extraction of protein from tissues, measurements of protein concentration, and western blot analysis of protein expression were performed as described previously (Wu, Divall, Wondisford, & Wolfe, 2012).AR antibody (N-20) and actin antibodies were purchased from Santa Cruz Biotechnology.

Hyperandrogenemic mouse model

Female mice were kept with normal chow and water ad libitum under a 14-h/10-h light/dark cycle. Creation of the DHT pellets followed previously described procedures (Andrisse et al., 2017; Xue et al., 2018). Dow Corning Silastic tubing (0.04 inch (1mm) inner diameter & 0.085 inch (2.15mm) outer diameter; Cat# 1118915D, Fisher Scientific, Hampton, NH) were filled with 4mm lengths of DHT or no-DHT, and then sealed with medical adhesive silicone (Factor II, Lakeside, AZ). Pellets were incubated in saline for 24 hours at 37°C for equilibration before insertion. Two months old female mice were subcutaneously implanted with a 4mm pellet with or without DHT. Pellets were replaced monthly to maintain a constant level of androgen excess. Experiments are conducted with hyperandrogenemic mice after 14 days implantation.

Assessment of puberty, estrous cyclicity, and reproductive performance in SynARKO females

Puberty was assessed starting on the 21st day of age by visual inspection of vaginal opening. After vaginal opening, the date of first estrous was determined by vaginal cytology (Wu et al., 2011). Estrous cyclicity was determined by performing a 16 day study of vaginal

cytology on SynARKO mice and -control littermates. Reproductive performance was assessed as previously described (Brothers et al., 2010). Briefly, each of four SynARKO females and four control females was mated with a proven fertile male (ARfl/y; Cre-) mouse for 90 days. The number of litters and the number of pups per litter were observed and documented.

Quantitative Real-Time PCR

Tissue from SynARKO and control mice was homogenized and RNA was collected using Trizol extraction (Invitrogen, Carlsbad, California, USA). RNA from AVPV, arcuate, hypothalamus, liver, ovary, muscle, and adipose tissue was reverse transcribed to cDNA. mRNA levels of genes of interest were measured on an I-Cycler quantitative PCR machine (Bio-Rad) using iQSYBR green reagent according to the manufacturer's protocol. 18S was used as an internal control. The genes tested were: AR (Androgen Receptor), GPR54 (G-Protein Coupled Receptor 54), KISS1 (KiSS-1 Metastasis-Suppressor).

Histology

Ovaries were dissected from SynARKO and control mice. Each sample was either snap frozen by liquid nitrogen and stored at -80 C or fixed in 10% formalin buffer. The tissues were submitted to the Johns Hopkins Histology Core Facility. The facility embedded the tissues in paraffin and sectioned them at 5 um thickness. Every 10^{th} section was collected and stained with hematoxylin and eosin (H&E). Ovaries were examined and follicles were counted with a Zeiss microscope.

Serum collection

Blood was collected from the mandibular vein via cheek puncture. Vaginal smear was used to determine the stage of the estrus cycle of the mouse at the time of the blood draw. Blood was centrifuged at 12,400 rcf for 15 min. The serum containing supernatant was stored at -80 C.

Hormone Assays

Morning levels (9-10 am) of LH and FSH from serum of mice at proestrus, estrus, and metestrus/diestrus were measured by luminex assay (Kit RPT-86K; EMD Millipore, Germany) Serum levels of estradiol were measured using an estradiol ELISA (Mouse E2 Elisa, Calbiotech,). Each estrous cycle stage was tested for estradiol levels. Serum testosterone levels were measured using testosterone ELISA (Total T Elisa, Calbiotech). LH levels were also tested by shipping 5ul to University of Virginia Ligand Assay Core (Charlottesville, Virginia).

MRI Body Composition Measurement

Body composition of the wild type and SynARKO mice was measured as described previously (Ma et al., 2017). Briefly, each mouse was loaded into the EchoMRI (EchoMedical Systems, USA) system to measure the fat, lean, and water mass. All measurements were made in the morning.

Body weight analysis

Mice were weighed every 7 days starting from day 14 through day 70. The cycling experiments and weight measurements were performed on the same group.

Indirect Calorimetry

After being allowed to acclimate to respiratory chambers for one day, mice were then monitored for 48 hours. Mice were kept under a 12-h light/ 12-dark cycle in a Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH).Data collected included rates of oxygen consumption, carbon dioxide production, respiratory exchange, heat, activity, body weight, and food intake.

Statistical Analysis

Each experiment was performed using either triplicate samples or duplicate samples (q-RT-PCR). We used an unpaired Student's t-test to compare the mRNA levels of genes between groups treated with low dose DHT or vehicle. All results are expressed as mean ± SEM and $p \leq 0.05$ assigned as significant using the GraphPad Prism 4 software (San Diego, CA, USA**).**

Results

Generation of SynARKO Mice

As diagrammed in Figure 4.1, we utilized cre lox recombinase technology in order to ablate AR expression specifically in central nervous systems expressing Synapsin Cre. AR fl/fl mice were generated by mating heterozygous female mouse (ARfl/wt; α Cre+/-) with male mouse (AR fl/ Y; α Cre-/-). Disrupted AR expression was determined by quantitative real-timePCR and western blot. AR mRNA levels were significantly reduced up to roughly 75% in hypothalamus of SynARKO mice compared with that in their control littermates. Other tissues such as the pituitary, ovary, and liver have similar levels of AR expression (Figure 4.2 A). Expression of AR is reduced in the SynARKO mice, but not abolished. AR protein levels were examined by western blotting (Figure 4.2B) and the protein levels were also significantly

reduced in hypothalamus of SynARKO mice compared with those in control mice; there was no change in AR protein expression levels in pituitary, ovary, and liver from control and SynARKO littermates (Figure 4.2),

SynARKO mice display same time of puberty and first estrus and similar pattern of estrous cyclicity

Vaginal opening and first estrus are two indicators of female puberty onset. Daily vaginal smears were obtained after vaginal opening to identify the age of first estrus. There was no significant difference in either vaginal opening or first estrus between the control and SynARKO groups (Figure 4.3 A). To assess the ongoing reproductive cyclicity of the female mice, vaginal cytology was analyzed from adult female mice for 16 consecutive days; control and SynARKO groups both exhibited regular estrous cycles (Figure 4.3 B,C). Although AR knocked out in CNS cells did not affect estrous cyclicity or fertility under normal physiological androgen level, we investigated if AR in CNS cells plays any role in hyperandrogenemia-induced acyclicity. We treated mice with 4-mm DHT pellets to produce serum androgen levels that mimic those in human hyperandrogenic phenotypes. We show that knocking out CNS AR does not rescue the acyclicity seen in DHT treated control mice. SynARKO-DHT and Con-DHT mice were both acyclic and spent comparable amounts of time in each estrous stage (Figure 4.3).

SynARKO female mice exhibited no difference in the numbers of litters compared to controls

Fertility was examined in a continuous mating protocol in control and SynARKO mice. Control or SynARKO female mice were mated with proven fertile male Control mice and the

total number of litters were recorded (Figure 4.4 A). During the 90-day mating period, there was no significant difference in the number of litters and pups between Control and SynARKO mice (Figure 4.4 B and C).

Hormone Levels are not altered in SynARKO mice

In order to determine if key hormone levels are altered in SynARKO mice, blood samples were collected in order to measure T, E2, LH, and FSH levels from adult female mice. Compared to control, there was no significant difference of T levels in SynARKO (Figure 4.5 A ,B) and E2 (Figure 4.5 C,D) levels at different cyclic stages or combined. During different estrous stages of the female mice cycle, LH (Figure 4.6 A) and FSH (Figure 4.6 B) levels were ascertained. While no difference in LH levels were seen between Control and SynARKO mice during different stages, FSH levels were similar only during the metestrus and proestrus stage. During the estrus stage, FSH levels were higher in Control female mice than in SynARKO.

Effect of CNS AR deletion on body weight and whole body composition

The central nervous system innervates the entire anatomy of mammals. Thus changes to the CNS physiology of a mouse could yield changes to the whole animal physiology. Total body weight was not different between Control and SynARKO female mice (Table 4.1). EchoMRI was conducted to measure total percent of fat and water. Treatment of Control and SynARKO female mice with DHT did not alter mice body composition (Table 4.1).

SynARKO mice with DHT have improved glucose tolerance compared to Control-DHT

Metabolic tests were conducted in order to determine the effect of CNS AR deletion on glucose homeostasis. At 4 months old and 2 months of DHT insertion a GTT and ITT protocol was performed (Figure 4.7 A-D). At 6-7 months old and 4-5 months of DHT insertion, the

same tests were repeated (Figure 4.7 E-H). After a 16 hour fast, female mice were injected with a bolus of glucose (2g/kg body weight) and their blood glucose levels were tracked for 120 minutes. At 4 months old (2 months of DHT insertion), SynARKO-DHT showed similar glucose tolerance compared to Control-DHT. (Figure 4.7 A, B). As expected blood glucose levels were significantly higher in DHT treated Control mice than untreated mice (Figure 4.7 A, B). Insulin resistance caused by HA is clearly evident in Figure 4.7 C and D. Both SynARKO-DHT and Con-DHT female mice serum glucose levels remained significantly higher over time in comparison to Con-no DHT after an injection of insulin (0.5Unit/kg). At 6-7 months old (4-5 months of DHT insertion) SynARKO-DHT showed improved glucose tolerance compared to Con-DHT (Figure 4.7 E, F). DHT induced insulin resistance was not corrected in SynARKO mice either at the early or later time points we performed the ITT (Figure 4.7 E,F).

DHT treated SynARKO mice have similar energy expenditure to untreated control mice

In order to determine the impact of CNS AR on whole body energy expenditure and other metabolic parameters, we performed indirect calorimetry analyses on Control and SynARKO mice using the CLAMS. Real-time monitoring showed VO2 consumption (Figure 4.8 A and B) and VCO2 production (Figure 4.8 C and D) were both decreased in DHT treated SynARKO mice compared to Control mice treated with and without DHT. Data from Figure 4.8 and Figure 4.9 are separated into dark and light cycles, due to known and well-studied behavioral difference of the mouse during the light and dark cycles. In Figure 4.9 A and B, the respiratory exchange ratio (RER) was calculated and was significantly increased in Con-DHT mice during light cycle. Although not significant, RER ratios of SynARKO-DHT mice were lower relative to Con-DHT mice in both light and dark cycles (Figure 4.9 A, B).

Furthermore the increase in body heat after DHT treatment in Control mice is attenuated in SynARKO mice (Figure 4.9 C and D). This was most evident in the light cycle. During the light cycle of locomotor activity (Figure 4.9 E) and food accumulation (Figure 4.9 G) a similar trend was observed in SynARKO-DHT mice. The trend being that SynARKO mice were protected from HA induced changes to energy expenditure. These data indicate a reduced metabolic rate and decreased energy expenditure after CNS AR deletion in female mice with DHT. To examine whether the difference observed in CLAMS were caused by change of body weight in SynARKO-DHT mice, we weighed the mice after CLAMS observation (Table 4.1 A). There was no significant difference in body weight between Con-DHT and SynARKO-DHT mice (Table 4.1 A), suggesting that metabolic and behavioral changes observed in SynARKO mice are not due to changes in body weight.

Discussion

Hyperandrogenemia (HA) has been shown to cause impaired glucose tolerance and insulin resistance in women and in female rodent models (Manneras et al., 2007; van Houten et al., 2012). The mechanism of this HA-induced metabolic dysfunction is not fully understood. We developed a mouse model (Figure 4.1) that displayed pathophysiological serum androgen levels with normal body mass (Table 4.1) to ensure that the phenotypes were directly from androgens effects and not due to the onset of obesity. It has previously been demonstrated in our laboratory as well as by other researchers that DHT causes impaired glucose, insulin and pyruvate tolerance, and lowered hepatic insulin action (Andrisse et al., 2017; Brothers et al., 2010; Yan et al., 2013; Wang et al., 2019) . The goal of this study was to determine the role of CNS AR in DHT-induced HA onset in female mice.

Infertility due to PCOS is a major problem affecting women of reproductive age all around the world. We hypothesized that CNS AR could mediate the HA induced infertility and acyclicity. We found no difference in age to puberty between Control and SynARKO female mice as indicated by vaginal opening (Figure 4.3 A). DHT is known to cause a reduction in numbers of estrous cycles as well as percent time spent in each stage. As shown in figure 4.3 B and C control mice treated with DHT showed reduced number of cycles and increased percent time in met/diestrus stage. SynARKO mice treated with DHT did not display a rescue of the aforementioned reductions. A mating study (Figure 4.4) conducted revealed no significant difference in the reproductive viability between Control and SynARKO female mice. This is unsurprising as Wu et al have previously demonstrated that it is the pituitary AR and or theca ovarian AR during HA, that is most likely responsible for normal reproductive function (Ma et al., 2017; Wang et al., 2019).

Findings from the Wu et al demonstrate that "androgens/AR signaling are important in the regulation of the preovulatory surge." This conclusion is supported by our own findings of altered and reduced FSH secretion in SynARKO mice in estrous (Figure 4.6 B). LH levels remained relatively unchanged between control and SynARKO mice (Figure 4.6A). Interestingly we saw no differences in *Gnrhr* mRNA expression (Figure 4.10 B). However when looking further upstream of the HPG axis at the hypothalamic expression of *Kiss1R,* we show that *Kiss1R* expression is reduced in the hypothalamus as well as the AVPV and Arcuate nuclei (Figure 4.10 A). The altered levels *Kiss1R* in SynARKO female mice could certainly lead to altered FSH secretion levels (Pineda, Aguilar, Pinilla, & Tena-Sempere, 2010; Thompson et al., 2004). Although FSH levels differed, there was ultimately no significant difference in blood serum estrogen and testosterone levels (Figure 4.5).

These previous findings taken together led us to conclude that physiologically, SynARKO mice are similar to control mice in reproduction and metabolic function. However, their responses to DHT induced HA were not similar when considering metabolic function. In order to determine the effects of CNS AR on metabolic homeostasis, we performed a GTT and ITT at two different time points. The "early stage" time point was at 4 months of age and a total DHT insertion time of 2 months. The "later stage" time point was after 6 months of age and 4 months of total DHT exposure. As previously mentioned, HA or treatment with DHT causes hyperglycemia. The effects of DHT was apparent at the early stage in Figure 4.7A and B as Control and SynARKO blood glucose levels were significantly increased after treatment with DHT. Remarkably, during the later stage of life, blood glucose levels of SynARKO-DHT female mice were significantly reduced in comparison to Con-DHT mice (Figure 4.7 E, F). SynARKO-DHT mice older than 6 months are seemingly protected from the increased blood glucose levels seen in Control-DHT mice after GTT (Figure 4.7 E, F).

An ITT was performed to determine if insulin resistance cause by HA is rescued via a CNS AR knockout. Not to our surprise, in the early stage of Control mice, DHT caused increased insulin resistance as indicated by the overall higher blood glucose levels after a bolus injection of insulin (Figure 4.7 C, D). At the "late stage" SynARKO and Control mice treated with DHT showed a more comparable amounts of insulin insensitivity, as shown in their high blood glucose levels after being challenged with insulin in figure 4.7 G and H. To our surprise, the improvement in GTT was not coupled with an improvement in insulin sensitivity in SynARKO-DHT groups. Possible explanations include insulin resistance in other tissues such as liver that lead to the increased blood glucose production. Altered expression of insulin

signaling molecules such as AKT, IR, GLUT, FOX-O1, and CREB between Control and SynARKO groups after DHT treatment could equally result in insulin resistance.

Defects in energy homeostasis and expenditure is an important indicator of metabolic syndrome (Mauvais-Jarvis, 2011). While the role of AR and HA on energy expenditure in males has been well studied (Fernando et al., 2010; Khaw & Barrett-Connor, 1992; Yu et al., 2008), the role of AR in female energy expenditure is not well known. We were able to analyze control and SynARKO female mice energy metabolism via a complete lab animal monitoring system (CLAMS). CLAMS revealed that after a 72 hour period of observation, DHT treated control mice consumed O2 and produced CO2 at the same levels (Figure 4.8 A and C). However, as quantified in Figure 4.8 B and D, SynARKO DHT treated mice showed significant decrease in O2 consumption and VO2 production versus DHT treated control mice.

In order to take into account the light effects on rodent energy expenditure, energy expenditure data is displayed in both the light and dark cycle. Measures of RER, heat, motion, and feeding all followed a similar trend as follows; DHT increases the aforementioned categories in control female mice, while SynARKO-DHT treated mice brought those levels back down towards those of Con-no DHT female mice (Figure 4.9). The ability of SynARKO to be protected from the DHT induced changes in energy expenditure was only apparent in the light cycle and not in the dark cycle. In the dark cycle all parameters of energy expenditure such as movement, food consumption and heat production were equal. This dramatic shift and reduction in energy expenditure in DHT treated SynARKO mice, leads us to believe that CNS AR plays a meditating role in HA induced metabolic and energy dysfunction. The ability of DHT treated SynARKO mice to behave as control untreated mice could also be reflected in their earlier noted improved GTT. The brain and spinal cord specific expression of AR in

cases of HA allows for the increased energy expenditure and imbalance noted in Con-DHT mice. However SynARKO-DHT female mice ameliorate these defects by protecting the mice from energy expenditure changes.

Our study reveals a crucial role for CNS AR on glucose and energy metabolism in middle- aged female mice with HA. Previous studies or AR deletion in the CNS such as that of Chang and colleagues was performed only in male mice and no GTT was conducted (Yu et al., 2013). To our knowledge, only one other group has attempted to delete brain expression of AR with HA in female mice. Caldwell et al, using a brain and hippocampus specific promoter (CamKII alpha cre) deleted AR in the aforementioned locations. After deletion of AR in the brains of female mice, Caldwell and colleagues then challenged 3 week old female mice named NeurARKO (Neuron Androgen Receptor Knock Out) with DHT pellets for 13 weeks. Mice were sacrificed at 16 weeks of age. NeurARKO mice treated with DHT did not develop anovulation, obesity, and dyslipidemia. Interestingly and similar to our findings, they were unable to rescue the acyclicity in these female mice caused by DHT treatment. As shown in Figure 4.3 B, Control-DHT and SynARKO DHT females had less than 1 total cycles. This lack of improvement in cyclicity after DHT treatment in both SynARKO and NeurARKO female mice leads us to believe that brain AR expression is not a target of HA induced acyclicity.

Another similar finding between female NeurARKO and SynARKO mice are that both groups still exhibited a high blood glucose after exposure to DHT before 5 months old. NeurARKO female mice as mentioned earlier were treated for 13 weeks with DHT and sacrificed at 16 weeks of age, which is relatively similar to our “early stage” time GTT of SynARKO mice. We saw no improvement in the glucose intolerance caused by DHT treatment in SynARKO mice at 4 months old, 2 months DHT insertion (Figure 4.7 A). However, unlike

the NeurARKO group, in which the female mice were sacrificed at 16 weeks (~4months), we noted an improved GTT in middle-aged female mice (6-7 months old, 4-5 months DHT insertion) who had been exposed to DHT for a far longer period of time (Figure 4.7 B). This improvement in glucose tolerance in "late stage" SynARKO mice was unable to be ascertained by Caldwell and colleagues most likely due to experimental design differences. It is possible that if they had utilized adult female mice instead of postnatal mice, as well as extended the time of DHT exposure, they also might have seen improved glucose and energy metabolism. Further noted differences between our study and that of Caldwell et al, include the dose of DHT used to induce HA. Our group used a low dose of DHT (≤2mg, 4mm length of pellet), while Caldwell and colleagues used a relatively higher dose of 10mg of DHT (1cm length of pellet) of DHT.

The brain as well as the CNS must now be recognized as sites of AR-mediated androgen action during HA. Here, using our low-dose DHT model of 4mm DHT pellets, we were able to induce HA in adult female mice and conclusively show that SynARKO female mice were protected from HA induced metabolic and energy dysfunction. Our results further demonstrate that CNS AR can be a target of treatment and future therapies to combat the devastating effects of PCOS in women.

Acknowledgements

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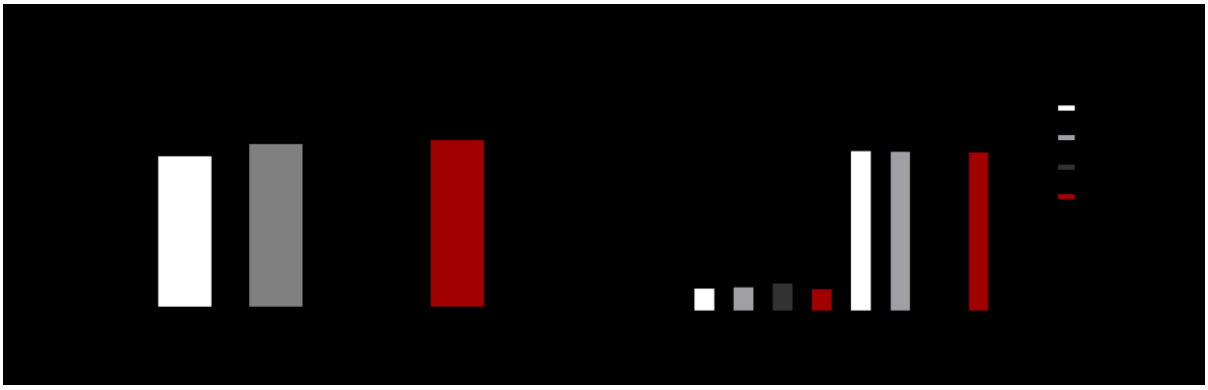
Table 4.1

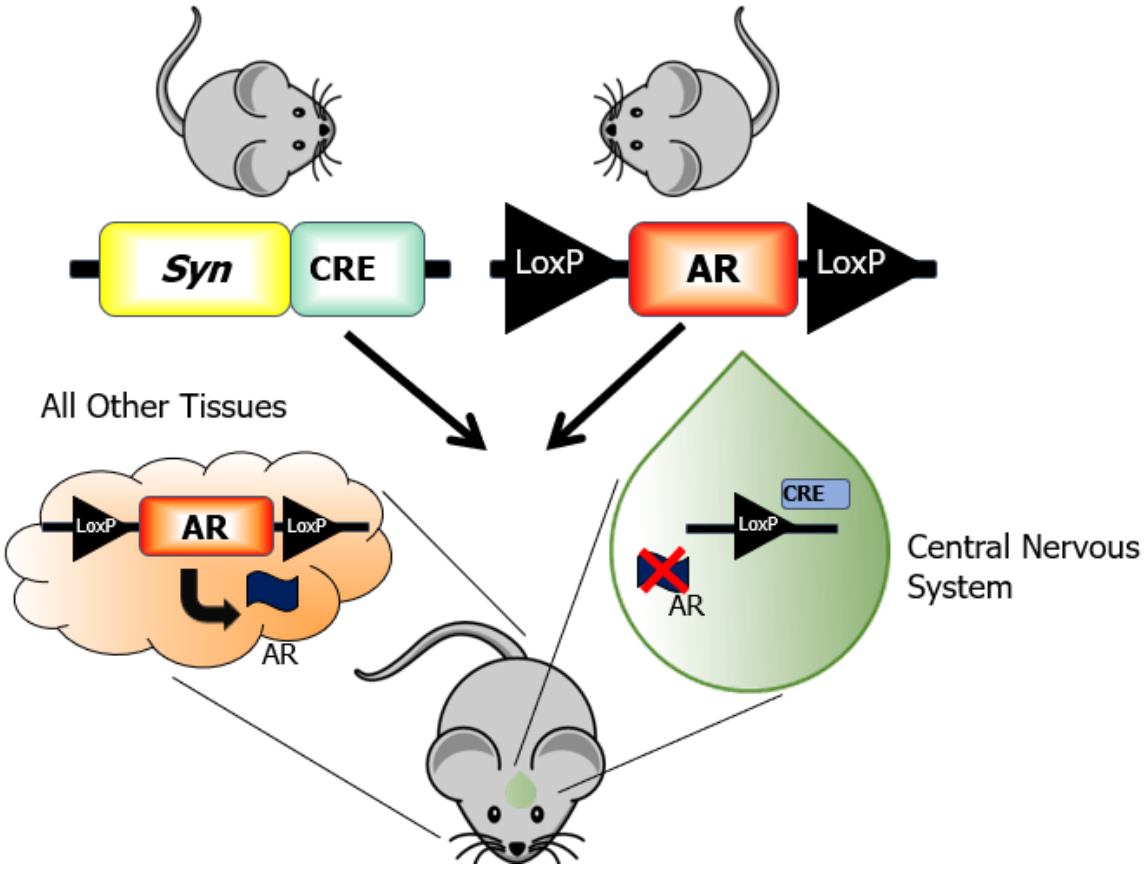
Body Composition of SynARKO Female Mice, 21 days after DHT insertion

	Weight(g)	Fat (%)	Lean (%)	Water (%)
Control	23.7 ± 0.7	11.95 ± 1.26	75.28 ± 1.01	60.95 ± 2.78
Control-DHT	24.1 ± .42	12.2 ±1.69	74.25 ± .86	59.26 ± 0.68
SynARKO	24.25 ±0.33	12.95 ± 1.09	73.96 ± 0.95	59.11 ± 0.45
SynARKO-DHT	23.45 ± 0.85	11.69 ± 1.62	74.68 ± 1.27	58.70 ± 0.93



Whole body composition analysis was performed using EchoMRI. Mice from control and SynARKO (KO) groups were treated with DHT before measurements 21 days later. **A.)** Body weight **B.)** Percent fat, lean and water were analyzed and quantified. Data were compared by One-Way ANOVA followed by Tukey's posthoc test. (n=4 mice per group)

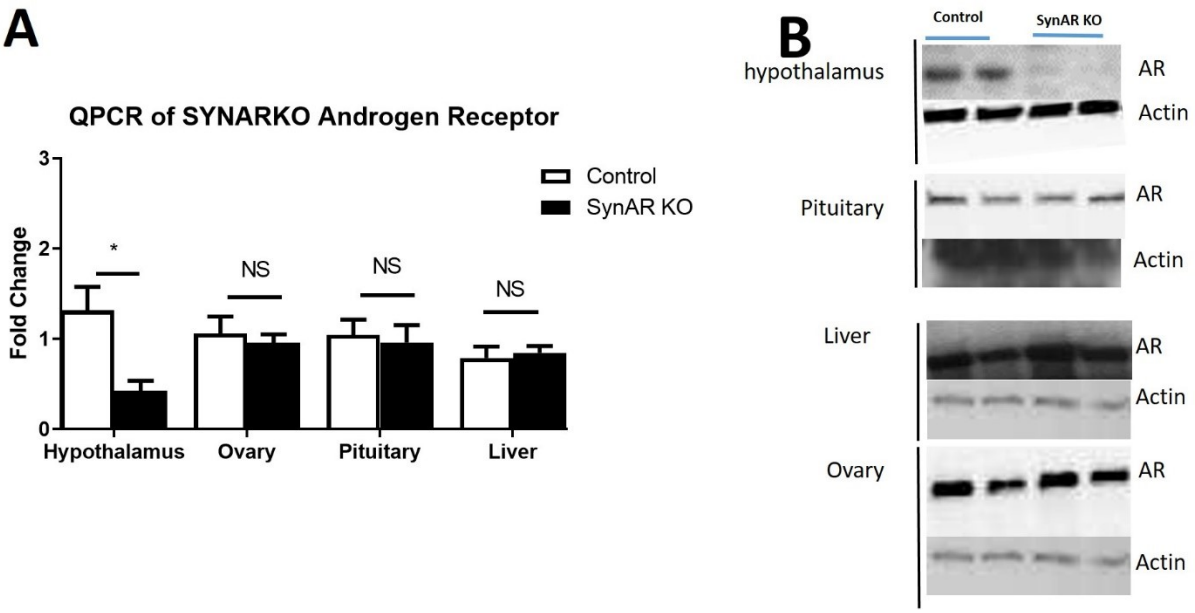
FIGURE4.1



Strategy for generation of SynARKO mouse

Schematic overview of generation of Syapsin Cre- Androgen Receptor KO Mouse using cre-lox recombinase technology. As shown, AR expression was specifically deleted in tissues of the central nervous system, while maintained in all other tissues.

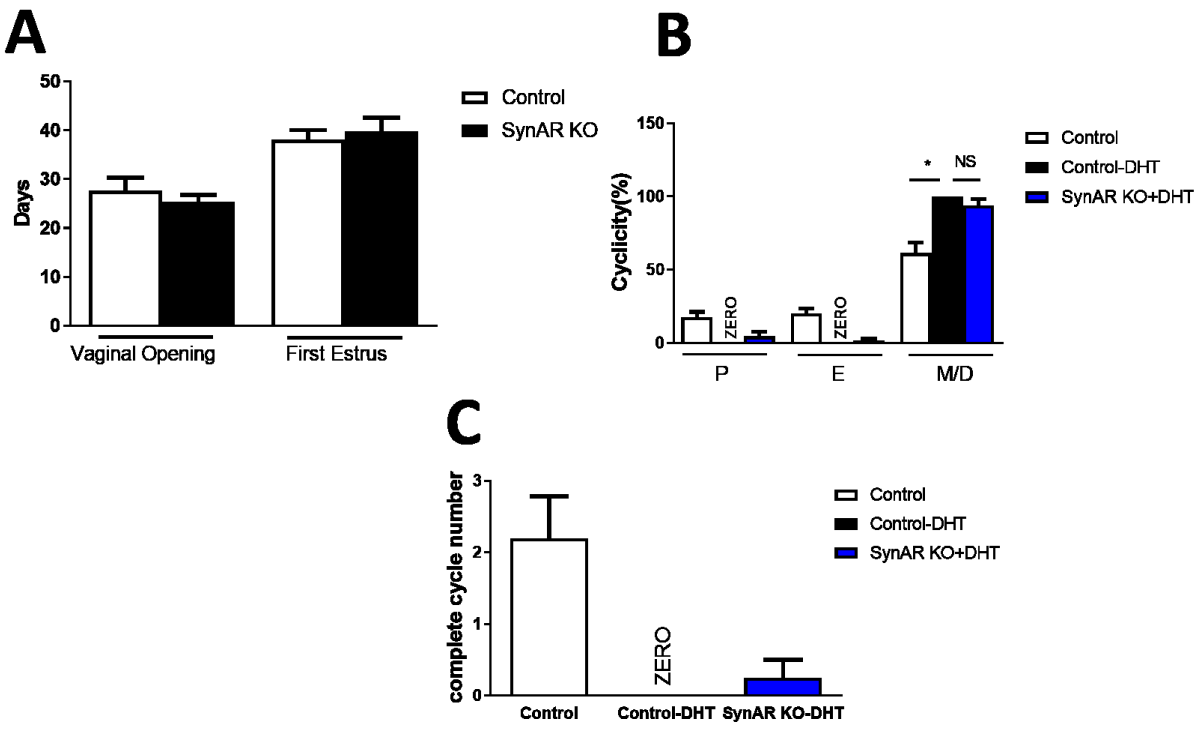
Figure 4.2



Confirmation of AR specific deletion in CNS

A.) Qpcr confirmation of central nervous system specific disruption of Androgen Receptor. AR is significantly reduced in the hypothalamus of the SynARKO mice. The AR mRNA level was measured by quantitative RT-PCR in 4 different tissues- hypothalamus, ovary, pituitary, and liver. AR was significantly reduced (75%) in the hypothalamus of SynARKO (KO) mice compared with that in control (Con) littermates, but no difference in AR expression was observed in other tissues. Data are means ± SEM (n = 6–20). **B.)** Protein confirmation of knockout. Actin protein was used as a loading control. Western blotting was performed, and AR protein levels were quantified by densitometry in 3 independent experiments. The AR protein level was reduced significantly in hypothalamus of SynARKO mice (B1 and B2), whereas no change was observed in other tissues compared with those in controls. Data are means ± SEM (n =6). *=P<0.05 as determined by a two tailed Students T-test.

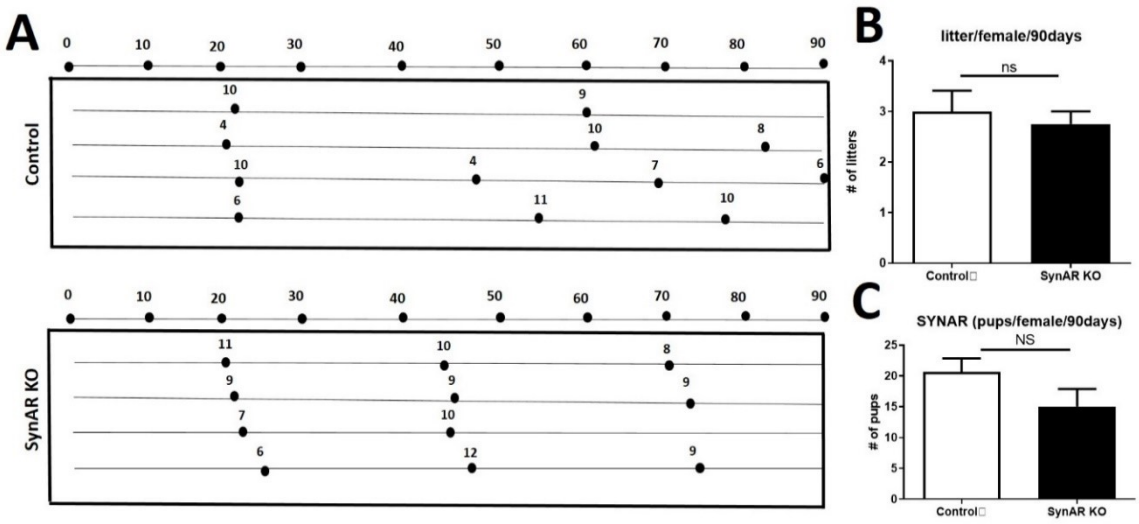
Figure 4.3



SynARKO mice display same time of puberty and first estrus, and similar pattern of estrous cyclicity with HA

Female puberty and cyclicity **A.)** SynARKO females exhibited an age of puberty onset similar to that of control (Con) littermates (n = 10–16). **B.)** Percentage of time spent in each stage was not significantly different between control and SynARKO mice when treated with DHT (n =10–21). **C.)** The number of complete cycles was not significantly different between control and SynARKO mice when treated with DHT (n =10–21). P, proestrus; E, estrus; M, metestrus; D, diestrus. Values are means ± SEM. *=P<0.05 as determined by a two tailed Students T-test or one way ANOVA followed by Tukey's *post-hoc* test.

Figure 4.4



SynARKO female mice exhibited no difference in the numbers of litters compared to controls

A.) Control and SynARKO female mice were introduced with a proven fertile male Control mouse, and the total numbers of litters per female were recorded during the 90 days (n =4).. **B.)** No difference in number of litters per female between control and SynARKO female. NS, no significant difference. **C.)** No difference in number of pups per female between control and SynARKO female. Values are means ± SEM relative to the control group as determined by a two tailed Students T-test.

Figure 4.5

Hormone levels are not altered in SynARKO mice

A.) and **B.).** Testosterone levels were measured at different stages of the estrous cycle (diestrus, proestrus and metestrus). There were no significant differences between control and SynARKO groups at each cyclic stage (A), and at combined stages (B) (n= 6–20). **C.)** and **D.)** E2 levels were similar between control and SynARKO groups of diestrus, proestrus and metestrus (C) and at combined stages (D) (n =6–14). NS, no significant difference. Values are means ± SEM relative to the control group as determined by a two tailed Students T-test.

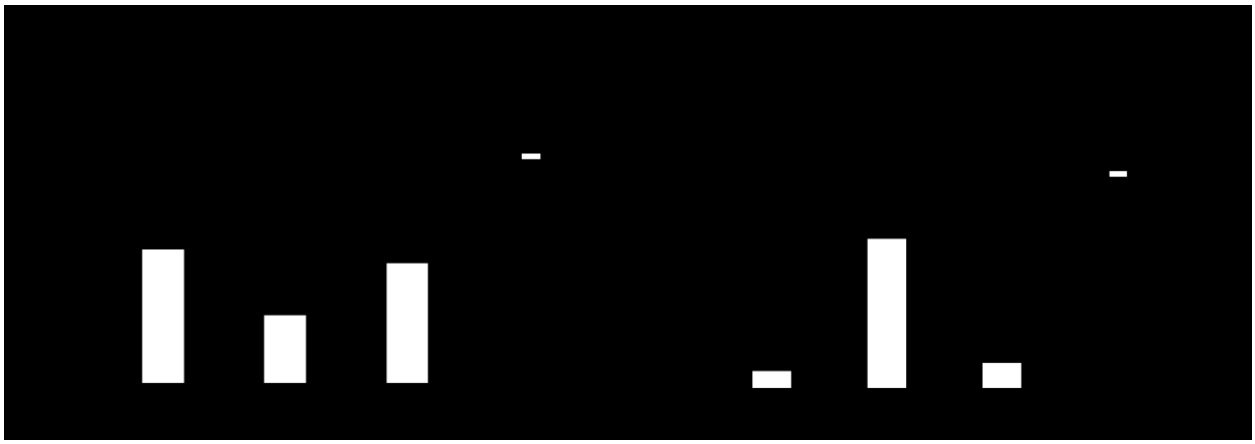
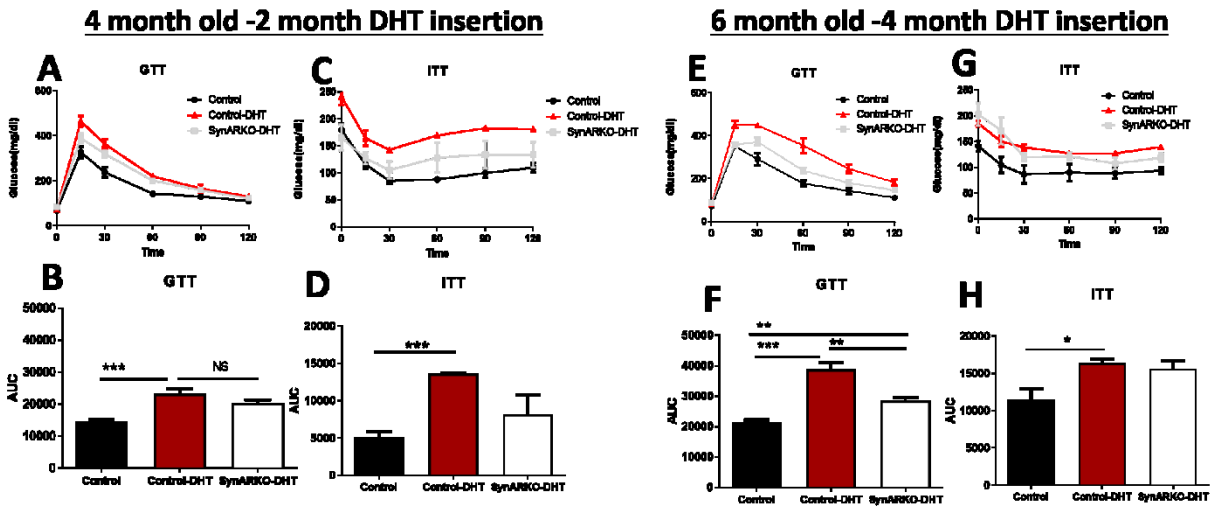


Figure 4.6

LH/FSH levels are not altered in SynARKO mice

A.) LH levels were measured during proestrus, estrus and metestrus. There was no change of LH levels in SynARKO mice compared with that in control mice at all three stages. **B.)** FSH levels were increased in SynARKO female mice during proestrus but significantly reduced during estrus when compared to control female. (n =8–15). Values are means ± SEM. *=P<0.05 relative to the control group as determined by a two tailed Students T-test.

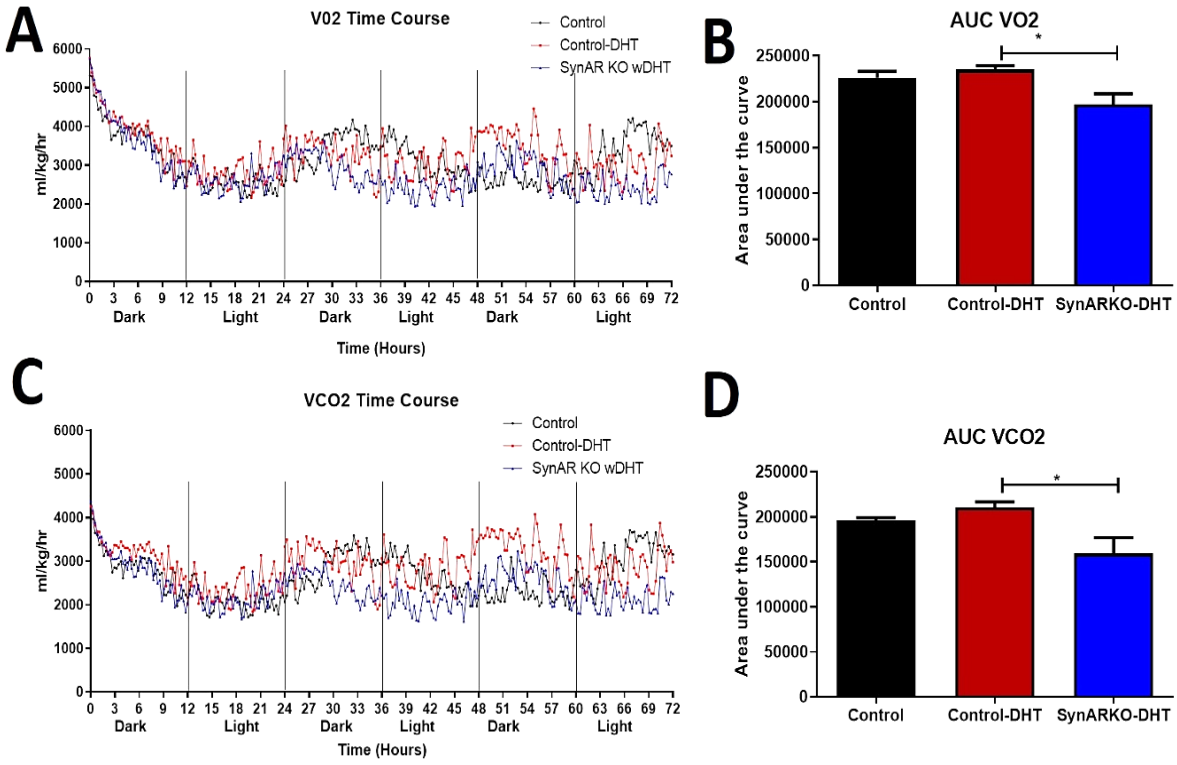
Figure 4.7



SynARKO mice with DHT have improved glucose tolerance compared to con-DHT

A) Glucose tolerance test (GTT) and **B)** area under the curve of GTTof 4-5 month old mice, **C)** insulin tolerance test (ITT) and **D)** area under the curve of ITT of 4-5 month old mice. Control, Control-DHT, and SynARKO-DHT mice were subjected to **(A)** a 16-hour fasted, 2 g/kg BW intraperitoneal GTT (n = 8 per group), **(C)** a 12-hour fasted, 0.3 U/kg BW ITT (n = 7 per group); E-H were mice at 6-7 month old with the same test as the mice at 4-5 month old. Values are means ± SEM . *=P < 0.05, **=P < 0.01, ***=P < 0.001 relative to the control group as determined by a two tailed Students T-test or one way ANOVA followed by Tukey's *post-hoc* test.

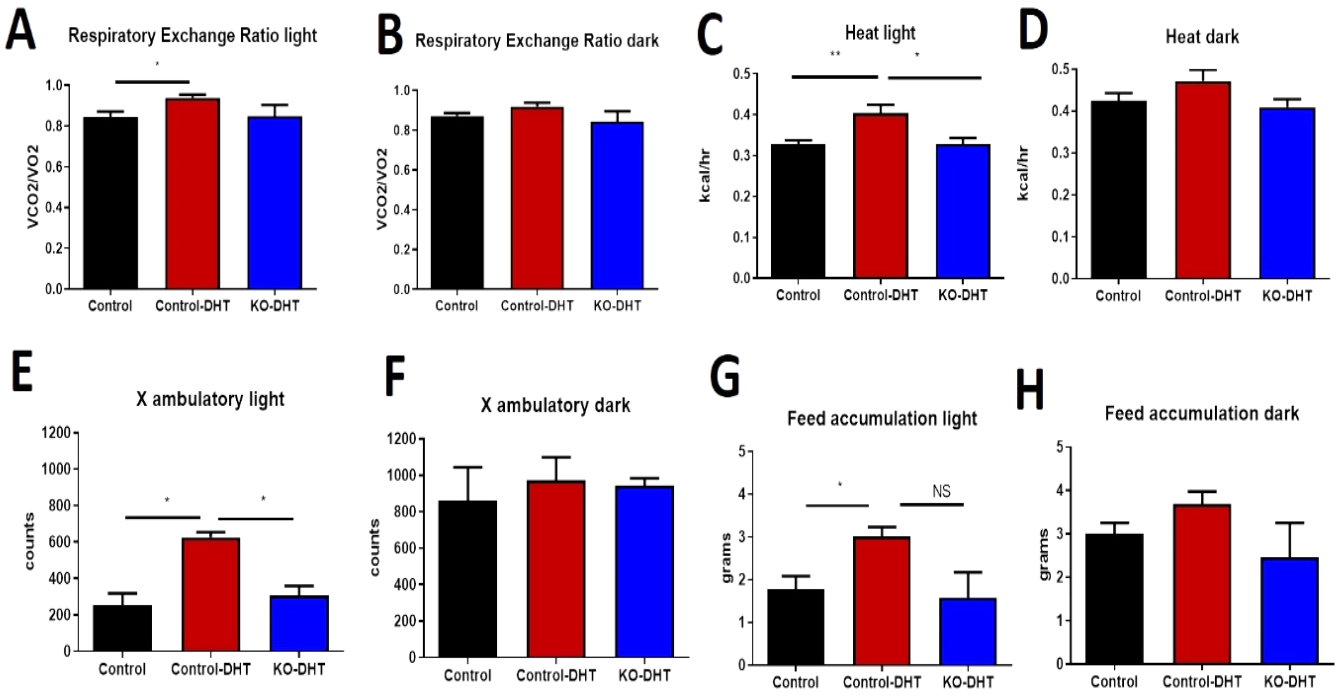
Figure 4.8



DHT treated SynARKO mice have similar energy expenditure to untreated control mice

A.) Real-time monitoring curve of oxygen consumption (VO2). **B.)** Quantification of O2 consumption. **C.)** Real-time monitoring curve of carbon dioxide release (VCO2). **D.)** Quantification of carbon dioxide release. Values are means ± SD. *$p < 0.05$ relative to the control group as determined by a two tailed Students T-test.

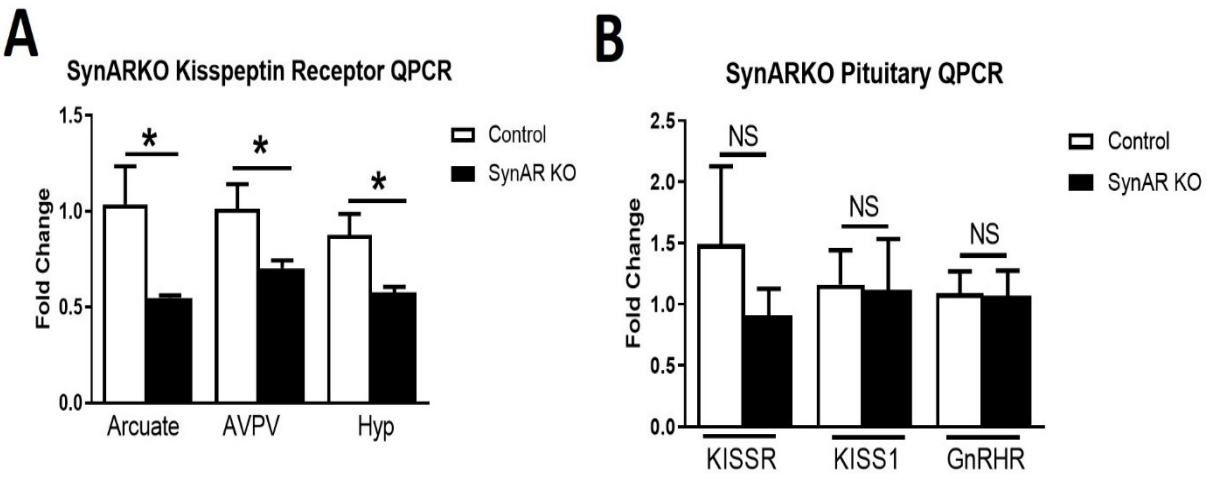
Figure 4.9



DHT treated SynARKO mice have similar energy expenditure to untreated control mice

A.) and **B.)** Quantification of real-time monitoring curve of respiratory exchange ratio (RER = VCO2/VO2) during the light and dark cycle. **C.)** and **D.)** Quantification of calculated body heat during the light and dark cycle. **E.)** and **F.)** Quantification of locomotor activity during the light and dark cycle. **G.)** and **H.)** Quantification of calculated accumulated food intake during the light and dark cycle. *P < 0.05,**P < 0.01, ***P < 0.001 relative to the control group as determined by a two tailed Students T-test.

Figure 4.10



A.) Kisspeptin Receptor mRNA levels were significantly reduced in the Arcuate and AVPV nuclei, as well as the hypothalamus of female SynARKO mice. **B.)** Important genes of pituitary function (KISSR, KISS1 and GnRHR) were measured by q-RT-PCR. Values are means ± SD. *$p < 0.05$ relative to the control group as determined by a two tailed Students T-test.

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EDUCATION

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2.) **Morehouse College -**Bachelor of Science- Biology & Public Health Minor; May 2013

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RESEARCH EXPERIENCE

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2.) **Undergraduate Researcher-Morehouse School of Medicine- Cardiovascular Research Institute (August 2010 –May 2013)**

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3.) **Research Intern- The Leadership Alliance, Columbia University Medical Center, (June 2011 – August 2011)**

Mentor- Mentors: Dr. Henry Ginsberg and Dr. Gissette Reyes-Soffer

4.) **Research Intern- University of Louisville School of Medicine, Department of Physiology and Biophysics Summer 2010**

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PUBLICATIONS

1.) Wang, Z., Feng, M., **Awe, O**., Ma, Y., Shen, M., Xue, P., . . . Wu, S. (2019). Gonadotrope androgen receptor mediates pituitary responsiveness to hormones and androgen-induced subfertility. JCI Insight doi, 10.1172/jci.insight.127817.

2.) **Awe, O.,*** Ma, Y., Wolfe, A., (2017) Exploring the Role of Pituitary Kisspeptin Receptor on Gonadotrophic Function in Vivo and In Vitro. The FASEB journal Published Online: 1 Apr Abstract Number: 714.1.

3.) Mishra, P., **Awe**, **O.,** Metreveli, N., Quipshidze, N., Joshua, I., Tyagi, S(2011) Exercise mitigates the homocysteine -beta2-adrenergic receptor interactions to ameliorate contractile dysfunction in diabetes. Int. Journal of Physiology, Pathophysiology, and Pharmacology; 3(2):97---106.

TEACHING/MENTORING EXPERIENCE

1.) Teaching Assistant (TA) for Scientific Foundations of Medicine, Cell Physiology Johns Hopkins University School of Medicine Baltimore, MD October- November 2016

2.) Morgan State University Baltimore, MD "A Student-Centered, Entrepreneurship Development) ASCEND Program May 2018- September 2018

3.) Students Mentored in Laboratory at Johns Hopkins University School of Medicine Baltimore, MD
   a. Melaku Arega, 4th year Undergraduate Johns Hopkins, 2/2/2014- 4/10/2016. 4th year Medical Student at Harvard Medical School
   b. Nazrawit Retta, 2nd year Undergraduate Johns Hopkins, 6/2/2016- 5/15/2017. 4th year Neuroscience Major.
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   d. Prerena Chatty. Johns Hopkins Medical Institute (JHMI) Student Internship Program. 6/1/2015-8/1/2015. Undergraduate at Cornell University.
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ABSTRACTS/ORAL PRESENTATIONS

- Johns Hopkins School of Medicine Research Seminar 2019 "A role for kisspeptin receptor in the pituitary gonadotroph in male mice."
- Experimental Biology Conference 2018 San Diego, CA "A role for kisspeptin receptor in the pituitary gonadotroph in male mice."
- American Physiological Intersociety Meeting, "Integrative Biology of Exercise "Westminster, CO.2012 "Role of β2-Adrenergic Receptors in T2Diabetes Using a Db/Db Mice Model System"
- John H. Hopps Symposium-Morehouse College-Atlanta, GA, September 2012 **"**The Role of Vitamin D supplementation in the progression of vascular cognitive impairment"
- Leadership Alliance National Symposium (LANS) in Old Greenwich, Connecticut July, 2011. "Lipoprotein Metabolism in Low Triglyceride vs. High Triglyceride Post-Menopausal Obese Women."
- Annual Biomedical Research Conference for Minority Students,-St. Louis, MO, November 2011 "Role of β2-Adrenergic Receptors in T2Diabetes Using a Db/Db Mice Model System'

AFFILIATIONS AND PROFESSIONAL SOCIETIES

- VP of General Affairs-Johns Hopkins School of Medicine Graduate Students Association (2015-2016
- Co-Chair, Physiology Department Research Seminars (2015-2016)
- American Physiological Society (2014- Present)
- Johns Hopkins School of Medicine –Biomedical Scholars Association (2014-Present)
- Phi Beta Kappa (2008-Present)
- Founder/President of Atlanta University African Students Association (2010-2012)
- Morehouse College Soccer Club (2009-2013)

HONORS & FELLOWSHIPS

- 2018 APS Travel Fellowship Award-Experimental Biology San Diego, CA
- 2016-2018 American Physiological Society- Porter Physiology Fellowship Recipient
- 2018 Johns Hopkins Graduate Student Association Travel Award for APS Comparative Physiology: Complexity & Integration Meeting New Orleans, LA.
- 2009-2013 President Scholarship from Morehouse College
- 2012 APS/NIDDK Minority Travel Fellowship Award for APS Intersociety Meeting, "Integrative Biology of Exercise "Westminster, CO.
- 2010 Outstanding Poster Presentation Award, Physiological Section, Annual Biomed. Research Conf. for Minority Students. Charlotte, NC.
- Star Scholarship from Montgomery College (2007-2008)